"EL SISTEMA UBIQUITINA-PROTEASOMA REGULA EL DESTINO DE LAS CÉLULAS TRONCALES MUSCULARES"

Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias Biológicas con mención en Biología Celular y Molecular.

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Enero de 2015.

Página de aprobación por el Comité de la Tesis

A mis abuelos Nelly y Francisco

Agradecimientos y financiamiento.

Quisiera agradecer en primer lugar a la Pontificia Universidad Católica de Chile, a sus funcionarios y alumnos. Esta universidad fue mi casa ya por más de diez años en los cuales descubrí mi vocación por la investigación científica. Me siento orgulloso de mi formación y afortunado por las personas que pude conocer entre sus paredes. Específicamente agradezco a la Vicerrectoría de Investigación UC y a la Dirección de Postgrado FCB por apoyo económico y por contribuir con mi formación integral como doctor. Especialmente agradezco a los funcionarios María Teresa Pino, Elizabeth Ly, Sylvia Soto, Lucy Chacoff, Héctor Casanova, Soledad García y Bárbara Salazar que tanta ayuda entregan para el desarrollo de nuestras investigaciones.

Agradezco infinitamente a las fuentes de financiamiento. Este trabajo fue financiado por las Becas CONICYT "Beca CONICYT para estudios de doctorado en Chile", "Beca apoyo a la participación en reuniones de sociedades científicas nacionales", "Beca de pasantía doctoral en el extranjero. Becas Chile", "Beca de apoyo a la realización de tesis doctoral" y "Beca de Asistencia a Eventos y Cursos Cortos para estudiantes de doctorado". También por la beca de la comisión Fulbright "Beca de pasantía doctoral en Estados Unidos". Esta tesis también fue financiada en gran parte por el proyecto FONDECYT 1130631 del Dr. Hugo Olguín.

Agradezco a mi tutor de tesis Hugo Olguín, con quien emprendimos el desafío de montar un laboratorio en condiciones adversas, pero con la firme convicción de que la ciencia bien realizada nos daría frutos. A nivel científico le agradezco sus buenos consejos basados en la experiencia para superar dificultades técnicas y también por su responsabilidad en lo mucho que he ganado en rigurosidad científica. Le doy gracias por mostrar humanidad ante las situaciones personales y por estar dispuesto a enseñarme protocolos directamente. Agradezco a la Dra D. Cornelison también por su excelente trato y preocupación durante mi pasantía doctoral en los Estados Unidos y en adelante hasta el día de hoy.

A mis compañeros de laboratorio les agradezco su compañía y colaboración sin las cuales el desarrollo de esta tesis no hubiese sido posible. A Marco Araya le agradezco por llegar a ser mi amigo y por interesarse en mis problemas, me siento orgulloso de haber formado parte importante de su crecimiento y ser testigo de lo que ha llegado a ser. A Natalia González le agradezco su buena disposición por ayudar siempre sin importar cuanto esto interrumpiera su propio avance, junto con esto me siento gratificado por haber podido ayudar en su formación como una gran bioquímica de la cual me considero su amigo. A Eduardo de la Vega y Felipe Cabezas les agradezco mucho por haber decidido aventurarse a llegar a nuestro laboratorio, creo que son un gran aporte en todo sentido, en el personal agradezco la preocupación de Eduardo por los demás y compañerismo que lo hacen una persona con la que uno puede contar y a Felipe por consejos técnicos con fundamentos de mucha utilidad. Creo

que el laboratorio tiene mucha suerte de contar con ambos para apuntar a lograr grandes cosas. A Sebastián Pérez le agradezco por ser una gran persona y un gran amigo, destaca su capacidad de escuchar e preocuparse de los demás desinteresadamente. A Gloria Loyola le agradezco por el cariño que entrega y por todo lo que pude aprender de ella. Gracias también a Cristina Padilla y a Reinaldo Castillo, con los cuales pude compartir poco tiempo pero que enriquecieron el laboratorio con su forma de ser.

Agradezco también a mis amigos los gringos, los cuales fueron grandes personas que me ayudaron de forma inigualable durante mi estadía en Estados Unidos, gracias a Peyton Walker, Dane Lund, Danny Stark, Patrick McAnulty y a los no gringos Tiago Tezzotto y Eric Villalón por toda su ayuda.

A los compañeros de laboratorio a quienes pude ayudar a formar, gracias por entregarme esa oportunidad y ayudarme a aprender a enseñar. Entre ellos a Camila Ibarra, Alonso Quiroz, Benjamín Sánchez, José Tomás Zemelman, Deysha Poyser, Gabriela Fernández y Alfredo Araneda. Les deseo lo mejor en el logro de sus objetivos en sus respectivas carreras universitarias y espero haber contribuído a su vocación por investigar.

Gracias a los demás laboratorios del Departamento de Biología Celular y Molecular de la PUC. Creo haber visitado todos los laboratorios en busca de equipos, reactivos, comentarios o ayuda técnica y les agradezco a todos por su buena disposición a ayudarme. Esta tesis también es de ustedes. Especialmente agradezco a los jefes y laboratorios de Dr. Juan Larraín (Héctor Carrasco), Dr. Enrique Brandan (Víctor Troncoso), Dr. María Paz Marzolo (Pamela Farfán), Alejandra Álvarez (Dr. Marcelo González), Dra. Pilar Carvallo, Dra. María Estela Andrés, Dr. Alfonso González (Dra. Andrea Soza) y Dr.Felipe Court.

Agradezco finalmente a Valentina Ayala, por todo su amor, apoyo, confianza y entrega desinteresada hacia mí, por atreverse y tener la valentía de vivir una vida conmigo, caminar de mi mano y por llevarse el peso de mi dedicación al laboratorio durante etapas muy complicadas de este proceso. Gracias por estar conmigo siempre y aunque a veces estuve lejos siempre fuiste el gran soporte que me permitió seguir adelante. *"Donde tu estás yo tengo el norte y no hay nada como tu amor como medio de transporte"*.

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LISTA DE ABREVIATURAS.

MRF: Muscle regulatory factor (Factor regulatorio muscular)

Pax7: Paired box protein 7 (Proteína de caja pareada 7)

UPS: Ubiquitin proteasome system (Sistema ubiquitina-proteasoma)

Nedd4: Neurally developmentally downregulated 4 (Disminuído durante el desarrollo neural 4)

Id3: Inhibitor of DNA-binding protein 3 (Proteína inhibidora de la unión al ADN 3)

Myf5: Myogenic factor 5 (Factor miogénico 5)

MyoD: Myogenic differentiation protein (Proteína de diferenciación miogénica)

RFP: Ret finger protein (Proteína con dedos Ret)

RING: Really interesting novel gene (Gen nuevo realmente interesante)

HECT: Homologous to E6AP Carboxy terminus (Homólogo a l carboxilo terminal de E6AP)

SC: Satellite cell (Célula satélite)

IF: Inmunoflurescencia

TAP: Tandem affinity purification (Purificación de afinidad en tándem)

MuDPIT: Multidimensional Protein Identification Technology (Tecnología de identificación de proteínas multidimensional)

BiFC: Bimolecular Fluorescence complementation (Complementación fluorescente bimolecular)

HA-tag: Human influenza hemagglutinin tag (epítope dela hemaglutinina de la influenza humana)

TA: *tibialis anterior*

LN: Laminina

GST: Glutatión S-transferasa

CRM1: Chromosome region maintenance 1 (mantención de la región del cromosoma)

LMB: Leptomicina-B

Pax3: Paired box protein 3 (Proteína de caja pareada 3)

Rpn: Regulatory particle non- ATPase (Partícula regulatoria no ATPasa)

TAP-CAT: Tandem affinity purification-tagged chloramphenicol acetyltransferase (Cloranfenicol acetiltransferasa marcada para purificación por afinidad en tándem)

FGF-2: Factor de crecimiento de fibroblastos 2

DMEM: Dulbecco's Modified Eagle Medium

AraC: 1-beta-D-arabino-furanosilcitosina

DMSO: Dimetil sulfóxido

RISC: RNA-induced silencing complex (Complejo de silenciamiento inducida por ARN)

PVDF: Polifluoruro de vinilideno

HRP: Horseradish peroxidase (Peroxidasa del rabanito)

IP: Inmunoprecipitación

UBE1: Ubiquitin-activating enzyme E1 (Enzima activadora de la ubiquitina 1)

UbcH5b: Ubiquitin-conjugating enzyme human 5b (Enzima humana conjugadora de la ubiquitina)

ERS: Energy regeneration solution (Solución regeneradora de energía)

ANOVA: Análisis de varianza

HD: Homeodominio

GFP: Green fluorescent protein (Proteína fluorescente verde)

GAPDH: Gliceraldehído 3-fosfato deshidrogenasa

MyHC: Myosin heavy chain (Cadena pesada de la miosina)

SUMO: Small Ubiquitin-like Modifier (Pequeño modificador similar a la ubiquitina)

RESUMEN.

Las células satélite son células troncales adultas tejido específicas del músculo esquelético. Fisiológicamente, estas células son las responsables de la gran capacidad regenerativa observada en el músculo. A nivel molecular, la diferenciación y autorrenovación de las células satélite está controlada por un balance delicado entre los factores regulatorios musculares que promueven la diferenciación y el factor de transcripción Pax7 el cual inhibe este proceso. Pax7 se expresa en las células satélite, sin embargo sus niveles proteicos disminuyen en las células que inician el proceso de diferenciación. Por otro lado, Pax7 es retenido en las células satélite generadas durante el proceso regenerativo por autorrenovación. Junto con esto, se ha demostrado que Pax7 controla la especificación, mantención y función de las células satélite *in vitro* e *in vivo*. Dadas las diversas funciones que posee Pax7 en las células satélite, los mecanismos involucrados en la regulación de la expresión de este factor constituyen una interrogante aún no resuelta y que se encuentra en la actual frontera del estudio de la biología de las células satélite.

Utilizando diversos modelos celulares, mediante aproximaciones de biología celular y molecular, experimentos farmacológicos y de ganancia y pérdida de función hemos investigado la función del sistema ubiquitina-proteasoma (UPS) en la regulación de los niveles de proteína de Pax7. El presente documento presenta los resultados obtenidos durante el desarrollo de mi tesis de doctorado, en la cual hemos demostrado que Pax7 es ubiquitinado y degradado por el proteasoma en mioblastos que inician el proceso de diferenciación muscular. Nuestros resultados indican que la enzima E3 ligasa Nedd4 media este proceso definiendo el destino celular de los precursores musculares. Estos resultados establecen por primera vez al UPS, específicamente a través de Nedd4, como un mecanismo que regula la expresión de Pax7 en las células satélite promoviendo la diferenciación muscular. La identificación de este mecanismo posee profundas implicancias para el estudio de las células satélite y su participación en la regeneración muscular durante la homeostasis o enfermedad.

ABSTRACT.

Satellite cells are tissue specific skeletal muscle adult stem cells. Physiologically, these cells are responsible for the remarkable muscle regenerative capacity. At the molecular level, self-renewal and differentiation of satellite cells is controlled by a delicate balance between muscle regulatory factors (MRFs) that promote the differentiation and Pax7 transcription factor, which inhibits this process. Pax7 is expressed in satellite cells. However, Pax7 protein levels decrease once differentiation process begins. Furthermore, Pax7 is retained in the satellite cells generated by self-renewal during muscle regeneration. Along with this, it has been shown that Pax7controls survival, proliferation, differentiation, identity and function of satellite cells *in vitro* and *in vivo*. Given the several Pax7 functions in satellite cells, the mechanisms involved in regulating Pax7 expression constitute an unresolved question which is in the current frontier of the research about satellite cells' biology.

Using various cell models, cellular and molecular biology approaches, pharmacological and gain and loss of function experiments we investigated the role of ubiquitin-proteasome system (UPS) in the regulation of Pax7 protein levels. This study presents the results obtained during this PhD thesis, in which we demonstrated that Pax7 is ubiquitinated and degraded by proteasome in myoblasts that enter muscle differentiation process. Our results indicate that Nedd4 E3 ligase enzyme mediates this regulation, which defines muscle precursors cell fate. These results establish for the first time UPS, specifically via Nedd4, as a mechanism that regulates Pax7 expression promoting muscle satellite cell differentiation. The identification of this mechanism has profound implications for the study of satellite cells and their function in muscle regeneration during homeostasis or disease.

INTRODUCCIÓN GENERAL

El músculo esquelético es un órgano crucial para la fisiología del organismo.

El músculo esquelético es el órgano encargado de funciones vitales para el organismo de los mamíferos y que corresponde a cerca del 40% de la masa corporal (Bowden, 1966). Está encargado de los movimientos reflejos, voluntarios e involuntarios. Este órgano contribuye al metabolismo energético y a la mantención de la temperatura (Homsher and Kean, 1978). Su función permite el ingreso de fuentes energéticas al organismo durante la obtención, masticación e ingestión del alimento, como también la salida de los desechos del organismo. Inclusive cuando está en reposo, el músculo esquelético juega un rol clave en el flujo sanguíneo y también responde con temblores involuntarios a las bajas temperaturas ambientales. Este órgano nos permite comunicar mediante la expresión facial, los gestos y el habla. También es fundamental para la reproducción y por tanto, para la supervivencia de la especie (Bowden, 1966).

El músculo esquelético está compuesto por fibras musculares multinucleadas. Los núcleos de estas células se encuentran cercanos a la membrana plasmática o sarcolema (Yin et al., 2013). El citoplasma de la fibra muscular está ocupado en gran proporción por el aparato contráctil, el cual está formado principalmente por fibras de actina y miosina. Esta organización permite su actividad como un sincicio coordinado para la contracción. Como consecuencia, las miofibras se encuentran en un estado post-mitótico, incapaces de proliferar. El músculo esquelético presenta además numerosos vasos sanguíneos, terminales nerviosas, células inmunes y tejido conjuntivo, los cuales actúan coordinadamente para permitir la

función muscular (Bowden, 1966). En el músculo esquelético además están presentes las células satélite (Mauro, 1961).

Las células satélite se requieren para la regeneración muscular.

El músculo esquelético posee una soprendente capacidad de regenerar frente a un daño, comparado con otros tejidos en el organismo adulto (Schiaffino and Partridge, 2008). Esta capacidad se explica por la presencia una población discreta de células satélite (Heslop et al., 2001; Moss and Leblond, 1970; Moss and Leblond, 1971). Estas células comprenden un 3-5% del total de núcleos en el músculo esquelético y se encuentran localizadas en un nicho característico entre la lámina basal y el sarcolema de las fibras musculares en un estado quiescente (Mauro, 1961; Schultz et al., 1978).

Las células satélite son consideradas células troncales debido a su capacidad de diferenciarse a miofibras y de en paralelo, autorrenovarse (Heslop et al., 2001; Moss and Leblond, 1971; Shi and Garry, 2006). Específicamente, las células satélite son células troncales unipotentes tejido-específicas del músculo esquelético. Fisiológicamente, las células satélite son las células encargadas de mantener y regenerar el músculo esquelético adulto (Lepper et al., 2011; Murphy et al., 2011; Relaix and Zammit, 2012; Sambasivan et al., 2011).

Pax7 es un factor de transcripción que posee funciones clave en la biología de las células satélite.

Pax7 es un factor de transcripción que pertenece a la familia de las proteínas Pax. Esta familia posee nueve miembros agrupados en base a homología de secuencia y a la presencia de un dominio pareado de unión al ADN. Los genes de la familia Pax han sido subclasificados

en cuatro subgrupos adicionales dependiendo de características estructurales. Componentes de esta familia juegan roles clave durante el desarrollo de los órganos y su mantención en el organismo adulto (Lang et al., 2007). Por otra parte, fallas en la función de proteínas de esta familia se relacionan con el desarrollo de distintas patologías, incluyendo el cáncer (Lang et al., 2007).

Pax7 une el ADN a través de un dominio de caja pareada presente en su amino terminal y dos del tipo homeodominio. Pax7 presenta un motivo octapeptido y el dominio de transactivación de Pax7 se encuentra en su carboxilo terminal (Schäfer et al., 1994). Existen cuatro variantes de splicing de Pax7 (Pax7a, Pax7b, Pax7c y Pac7d), los cuales difieren en uno, dos o tres aminoácidos en su dominio de caja pareada (White and Ziman, 2008). De las cuatro isoformas, Pax7d es la que se expresa en mayor proporción en las células satélite. Pax7 se une a secuencias consenso en el ADN del tipo homeobox y caja pareada (Soleimani et al., 2012) induciendo la activación de genes como como ID3 y CDC20 (Diao et al., 2012; Kumar et al., 2009).

Durante el desarrollo embrionario Pax7 se expresa en las crestas neurales y precursores del mesencéfalo (Jostes et al., 1990; Murdoch et al., 2012). Pax7 es también expresado durante el desarrollo muscular en progenitores miogénicos de la región central del dermomiotomo (Jostes et al., 1990; Lepper and Fan, 2010; Relaix et al., 2004) los cuales dan origen a un grupo particular de músculos de la región central del embrión y a las células satélite (Lepper and Fan, 2010; Relaix et al., 2005).

La expresión de Pax7 como un marcador de las células satélite en el músculo adulto, ha sido rápidamente asociada con diversas funciones. Pax7 es necesario para la especificación y

propagación de las células satélite (Oustanina et al., 2004; Seale et al., 2000). Ha sido demostrado que ratones transgénicos nulos para Pax7 poseen un número reducido de células satélite, las cuales desaparecen gradualmente durante el desarrollo postnatal. Las células satélite del ratón nulo de Pax7 presentan una menor capacidad de dar origen a mioblastos – células proliferativas comprometidas, descendientes de las células satélite- que las del ratón silvestre (Oustanina et al., 2004; Seale et al., 2000). Por otro lado, mediante experimentos de manipulación artificial de los niveles de Pax7, se ha demostrado que este factor posee funciones adicionales regulando la proliferación, diferenciación y morfología de los mioblastos derivados de las células satélite (Collins et al., 2009; Olguin and Olwin, 2004; Zammit et al., 2006; Zhuang et al., 2014).

El requerimiento funcional de Pax7 en la regeneración muscular ha estado en discusión en los últimos años. Ratones que son nulos para Pax7 presentan defectos en su regeneración muscular en respuesta a un daño agudo, indicando que Pax7 se requiere para la regeneración (Kuang et al., 2006; Oustanina et al., 2004; Seale et al., 2000). A pesar de esta evidencia, parecía difícil distinguir los efectos de la deleción de Pax7 durante la especificación de las células satélite de una función específica de este factor durante la actividad de las células satélite en el proceso regenerativo.

En paralelo al desarrollo de esta tesis de doctorado, se reportó una estrategia que ha contribuido al entendimiento de la función de Pax7 en la regeneración muscular. Se desarrollaron ratones transgénicos en los cuales el gen de Pax7 es removido de forma inducible específicamente en las células satélite del animal adulto (Lepper and Fan, 2012). Esta aproximación permitió demostrar que Pax7 es necesario para la función de las células

satélite en la regeneración muscular (von Maltzahn et al., 2013). Las células satélite en las que Pax7 es inactivado no proliferan y se ha observado que estas células ingresan ya sea en apoptosis o bien se diferencian precozmente. Dichos destinos resultan en falta de regeneración frente a un daño agudo (von Maltzahn et al., 2013).

Pax7 posee funciones dependientes e independientes de su actividad como factor de transcripción.

Se ha identificado algunos blancos transcripcionales de Pax7durante la miogénesis tales como los genes ID3, CDC20 Y MYF5 (Diao et al., 2012; Kumar et al., 2009; McKinnell et al., 2008). Sin embargo, Pax7 posee una reducida capacidad de transactivación en comparación a otras proteínas de la familia debido a dominios de represión en cis, presentes en su amino terminal y el homeodominio (Bennicelli et al., 1999). Por lo tanto, se plantea que interacciones con otras proteínas podrían regular su función transcripcional (Olguín and Pisconti, 2011). Por ejemplo, Pax7 interactúa con el complejo metiltransferasa de histonas Wdr5–Ash2L–MLL2 a través del dominio de caja pareada de Pax7. Este complejo molecular se une a *enhancers* presentes río arriba del promotor de Myf5 induciendo su expresión en precursores musculares y por tanto resultando en la entrada de estas células al compromiso miogénico (McKinnell et al., 2008).

Por el contrario, la posibilidad de que Pax7 actúe como un represor de la transcripción no ha sido estudiada en profundidad, sin embargo, experimentos de genoma global muestran que la sobreexpresión de Pax7 puede resultar en la represión de un conjunto de genes relacionados a la diferenciación muscular y que se existen secuencias consenso de unión a Pax7 en esos genes (Soleimani et al., 2012). Además resultados preliminares de nuestro

laboratorio indican que Pax7 podría interactuar con componentes del complejo represor NCoR, como Tblr1 y también con componentes del complejo represor mSin3a como ARID4B (Olguín HC, datos no publicados). La relevancia funcional de dichas interacciones en el contexto de las células satélite se encuentra en investigación.

Por otro lado, la capacidad de Pax7 de inhibir la miogénesis depende de su homeodominio (Olguin et al., 2007). Lo que indicaría que Pax7 activaría o inhibiría la miogénesis por mecanismos dependientes e independientes de su actividad como factor de transcripción, los cuales dependerían de distintos dominios de la estructura de Pax7 (Olguín and Pisconti, 2012).

El destino celular de los precursores musculares es controlado por una regulación cruzada entre Pax7 y los factores regulatorios musculares.

Los mecanismos moleculares mediante los cuales Pax7 regula la miogénesis representan una de las preguntas no resueltas en el campo de estudio de las células satélite (Kuang and Rudnicki, 2008). Como consecuencia, varios grupos han desarrollado diversos enfoques para intentar responder esta interrogante (Olguín and Pisconti, 2012).

Pax7 se expresa en las células satélite que se encuentran en reposo proliferativo (Seale et al., 2000), sin embargo, los niveles de proteína de Pax7 disminuyen rápidamente una vez que las células satélite son activadas y comienzan a diferenciarse (Halevy et al., 2004; Olguin and Olwin, 2004; Seale et al., 2000; Zammit et al., 2004). Este patrón de expresión de Pax7 se correlaciona con la inducción de los factores regulatorios musculares (MRFs), MyoD y miogenina cuya función resulta en la diferenciación terminal (Olguin and Olwin, 2004; Zammit et al., 2004). Sin embargo, las células que autorrenuevan la población de células

satélite, disminuyen los niveles de MyoD y mantienen los niveles de proteína de Pax7 (Zammit et al., 2004) (Figura I2).

MyoD es el gen maestro de la diferenciación muscular (Berkes and Tapscott, 2005). Este gen fue identificado por su capacidad de reprogramar células de diversos orígenes al linaje muscular cuando es expresado ectópicamente (Weintraub et al., 1989) y por tanto es suficiente para remodelar la cromatina e inducir la expresión de genes específicos del músculo esquelético (Berkes and Tapscott, 2005). MyoD heterodimeriza con los factores de transcripción proteínas E. Este dímero se une a las secuencias consenso Cajas-E, induciendo la expresión de miogenina y otros genes específicos, que controlan la diferenciación terminal del linaje muscular esquelético (Sabourin and Rudnicki, 2000).

Durante la miogénesis existe un circuito molecular regulatorio entre Pax7 y los MRFs. Se ha planteado que la conversación entre estos factores es clave para definir el destino de las células satélite y sus decisiones hacia el compromiso miogénico y la autorrenovación (Olguín and Pisconti, 2012). Pax7 inhibe la actividad de MyoD mediante la interacción entre ambas proteínas la que resulta en la ubiquitinación y degradación proteasomal de MyoD, proceso mediado por la E3 ligasa RFP (Joung et al., 2014; Olguin et al., 2007). Además Pax7 induce la expresión de Id3, un factor que inhibe la actividad de MyoD evitando su interacción con las proteínas E (Kumar et al., 2009). Por otro lado, MyoD inhibe la expresión de Pax7 mediante la inducción de micro ARNs, los cuales inhiben la traducción del mensajero de Pax7 (Chen et al., 2010; Dey et al., 2011).

Debido a la naturaleza de los mecanismos mencionados, por los cuales Pax7 se relaciona con los MRFs, se ha propuesto que pequeños cambios en el balance de los niveles de

proteína de estos factores sean determinantes para las decisiones celulares durante el desarrollo y regeneración (Olguín and Pisconti, 2012).

El sistema ubiqutina-proteasoma degrada proteínas celulares regulando su función.

El sistema ubiquitina-proteasoma regula una gran cantidad de procesos celulares durante la homeostasis, proliferación y diferenciación celular. Fallas en este sistema desembocan en diversas patologías incluyendo el cáncer (Petroski, 2008).

La ubiquitinación es un mecanismo celular de destinación de proteínas solubles o de membrana a su degradación o cambios en su localización o actividad (Hershko and Ciechanover, 1998). Esta reacción consiste en la ligación covalente de la proteína de 76 aminoácidos llamada ubiquitina a residuos de lisina específicos en la proteína blanco de acuerdo a los requerimientos celulares (Hershko and Ciechanover, 2006). La ubiquitinación es mediada por la actividad de tres enzimas que catalizan la transferencia secuencial de la ubiquitina a la proteína blanco. En primer lugar la enzima E1 activadora recibe la ubiquitina libre, luego la enzima E2 conjugadora se une transitoriamente a la ubiquitina y finalmente la enzima E3 ligasa cataliza la transferencia de la ubiquitina desde la E2 a un residuo de lisina en la proteína blanco (Pickart, 2001). La ubiquitinación ha sido clásicamente descrita como una señal de destinación de proteínas a su degradación por acción del complejo 26S proteasoma (Hershko and Ciechanover, 2006).

En las células eucariontes existe una isoforma de la enzima E1 activadora, aproximadamente 50 enzimas E2 conjugadoras y 500 diferentes E3 ligasas. Esta jerarquía indica que la E3 ligasa es la responsable de entregar especificidad de sustrato a la reacción. En

consecuencia la identificación de la E3 ligasa corresponde a un paso importante para entender la regulación de una proteína que es ubiquitinada (Pickart, 2001).

Las enzimas E3 ligasas se dividen entre dos grandes subgrupos, las E3 del tipo RING (*Really interesting novel gene*) y las E3 del tipo HECT (*Homologous to E6-AP Carboxy terminus*). Las enzimas del tipo HECT se caracterizan por poseer un residuo de cisteína en su dominio catalítico al cual se une la ubiquitina para luego ser transferida a la proteína blanco (Bernassola et al., 2008). El exponente más estudiado de las E3 del tipo HECT es Nedd4, enzima que cataliza la ubiquitinación de diversos sustratos en contextos celulares particulares, incluyendo proteínas de membrana, citoplasmáticos y nucleares (Harvey and Kumar, 1999; Yang and Kumar, 2010). Nedd4 se expresa en el músculo esquelético (Koncarevic et al., 2007; Kumar et al., 1997), sin embargo no se ha descrito su expresión y función en las células satélite.

Pax7 interactúa con proteínas que participan en sistema ubiquitina-proteasoma, lo que podría regular su estabilidad.

En base a la importancia funcional que posee Pax7 para el potencial regenerativo de las células satélite, los mecanismos que están involucrados en (i) la disminución de los niveles de proteína de Pax7 en las células que se diferencian, (ii) de la mantención de esta proteína en las células que se autorrenuevan durante la regeneración y (iii) la regulación de su función durante la miogénesis, constituyen preguntas relevantes en la actual frontera del conocimiento de la biología de las células satélite.

Se ha demostrado que la interacción de proteínas de la familia Pax con otras proteínas son críticas para su función (Cai et al., 2003; Nitsch et al., 2004; Sato et al., 2001). En el caso

de Pax7, su interacción con complejos metiltransferasas de histonas media su capacidad de inducir la transcripción de MYF5 (Diao et al., 2012; Kawabe et al., 2012; McKinnell et al., 2008) y de promover la proliferación de los precursores musculares. Esta interacción está mediada por una interacción previa de Pax7 con la arginina metiltransfereasa Carm1 la que media su interacción con el complejo (Kawabe et al., 2012). Además recientemente se ha demostrado que Pax7 interactúa con beta-catenina y dicha interacción resulta en la inhibición de la actividad de la vía Wnt y de la diferenciación de los mioblastos (Zhuang et al., 2014). Así también Pax7 interactúa con MyoD, factor que induce la expresión de miogenina, la cual promueve una reducción de los niveles de Pax7 que es dependiente de la actividad del proteasoma (Olguin et al., 2007). En base a estos antecedentes es posible que las interacciones con otras proteínas sean relevantes para modular la actividad de Pax7 como regulador de la miogénesis.

Con el objetivo de identificar nuevas proteínas que interactúan con Pax7 durante la miogénesis y también posibles candidatos a ser reguladores de Pax7 en este contexto, transdujimos células C3H10T1/2 con TAP-Pax7 y MyoD (Bustos et al., 2015). De tal forma de permitirnos identificar reguladores positivos y negativos de la expresión de Pax7, las células sometidas al ensayo expresaban niveles intermedios de proteína de MyoD y Pax7. Además, las células fueron tratadas con el inhibidor del proteasoma MG132 debido a que estaba reportado que este tratamiento estabiliza a Pax7 en mioblastos en diferenciación (Olguin et al., 2007). Los lisados celulares obtenidos fueron sometidos a TAP (Purificación de afinidad en tándem; del inglés: T*andem Affinity Purification*) acoplado a espectrometría de masas del tipo MudPIT (Tecnología de identificación multidimensional de proteínas; del

inglés: *Multidimensional Protein Identification Technology*) (Figura I1A) (Bustos et al., 2015).

Mediante este ensayo fue posible identificar diversas proteínas que interactúan con Pax7 (Fig. I1). Entre ellas destaca un grupo de proteínas correspondientes a componentes del sistema ubiquitina-proteasoma, entre las cuales destaca la presencia de la E3 ligasa Nedd4 (Figura I1B), puesto que la enzima E3 es la que entrega la especificidad de sustrato a la reacción de ubiquitinación. Como consecuencia y en base a los antecedentes aquí enunciados, planteamos la siguiente hipótesis (Figura I2) y objetivos de investigación:

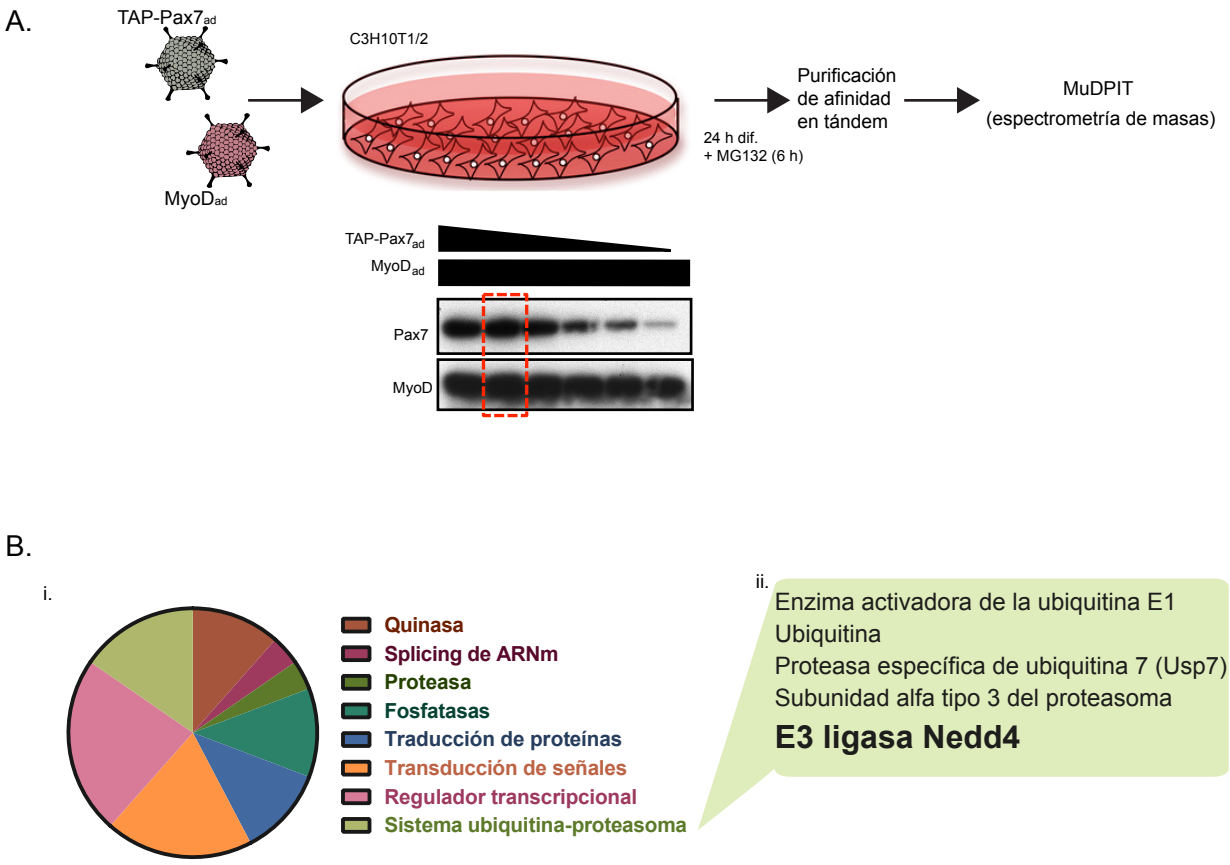

Figura I1: Identificación de proteínas interactuantes con Pax7 durante la miogénesis mediante espectrometría de masas MudPIT.

A. Esquema de la aproximación celular utilizada. Células C3H10T1/2 fueron transducidas con vectores virales para la expresión de TAP-Pax7 y MyoD, luego las células fueron inducidas a diferenciar por 24 horas y tratadas con el inhibidor del proteasoma MG132 a 25 μM las últimas 6 horas antes de la lisis celular. La expresión de Pax7 y MyoD fue confirmada mediante western blot y se escogió la muestra que presentaba un nivel de expresión proteico intermedio de Pax7 y MyoD (panel inferior cuadro rojo) para ser sometido a una purificación de afinidad en tándem y posteriormente a espectrometría de masas para la identificación de proteínas. B. (i) Pax7 interactúa con un grupo de proteínas clasificadas de acuerdo a su función en procesos de la célula (Gráfico de torta). (ii) Se destaca la presencia de un grupo de proteínas que interactúan con Pax7 en este ensayo pertenecientes al sistema ubiquitina-proteasoma (cuadro verde). Entre ellas, nuestro trabajo plantea a Nedd4 como una candidata a ser la E3 ligasa que cataliza la ubiquitinación de Pax7 en precursores musculares adultos.

HIPÓTESIS:

"El sistema ubiquitina-proteasoma a través de la E3 ubiquitina ligasa Nedd4 regula la degradación de Pax7, en precursores musculares que inician el proceso de diferenciación terminal"

OBJETIVO GENERAL:

Determinar y caracterizar la función del sistema ubiqutina-proteasoma y la E3 ligasa Nedd4 en la regulación de la ubiquitinación y degradación de Pax7, durante las etapas tempranas de la diferenciación muscular.

OBJETIVOS ESPECÍFICOS:

1- Caracterizar la regulación de la estabilidad de Pax7 mediada por el UPS durante la diferenciación de los progenitores musculares adultos.

2- Estudiar la interacción bioquímica y funcional entre Nedd4 y Pax7, en mioblastos adultos.

3- Delinear mecanismos involucrados en la regulación de la función de Nedd4 en el control de la estabilidad de Pax7 y su efecto en la función de progenitores musculares adultos.

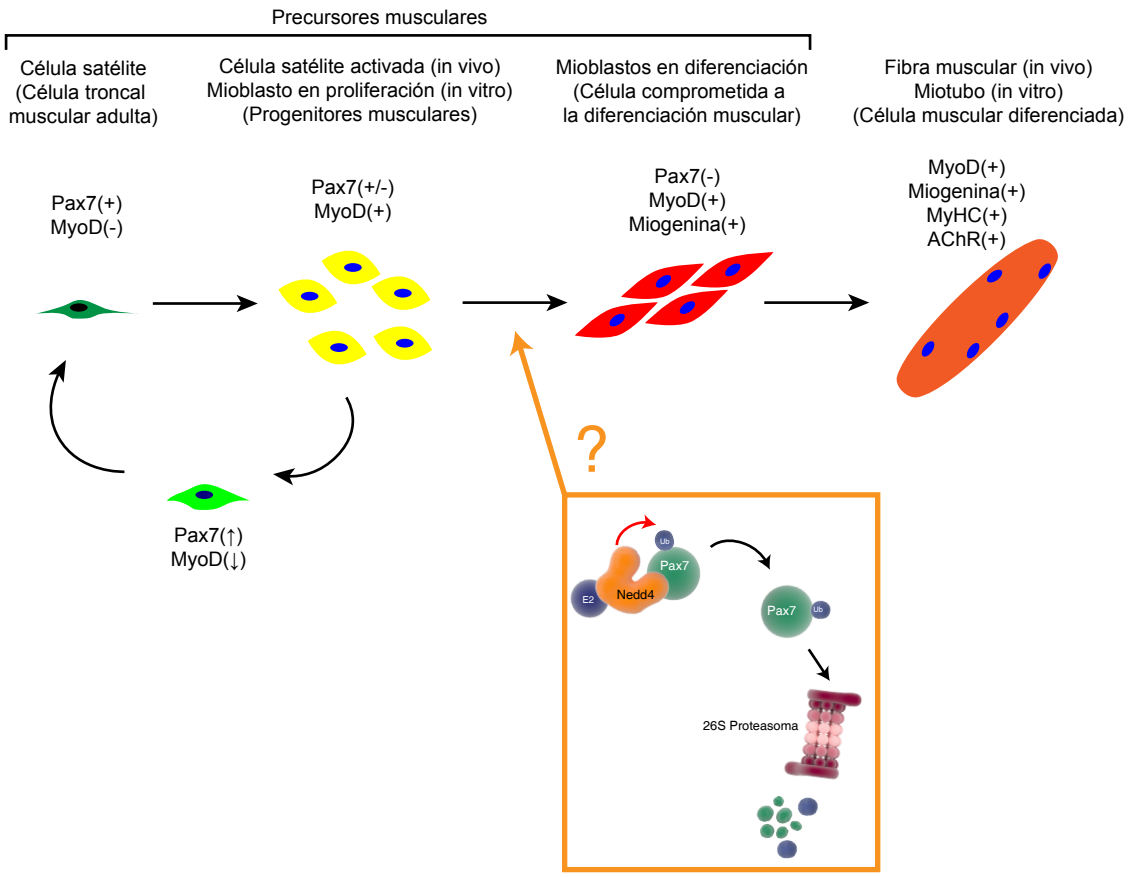

Figura I2: Hipótesis de trabajo.
Arriba: Esquema de la diferenciación de las células satélite durante el proceso de regeneración muscular (miogénesis adulta). Se indican los términos utilizados para nombrar a las células satélite y sus descendientes en las distintas etapas de la diferenciación *in vivo* o en modelos *in vitro*. Se muestra también la presencia o ausencia de marcadores moleculares específicos de las etapas de la diferenciación ilustradas (Adaptado de Olguin et al., 2007). Cuadro inferior: En base a evidencia previa nuestra hipótesis de trabajo plantea que la E3 ubiquitina ligasa Nedd4 regula la degradación de Pax7 a través del sistema ubiquitina proteasoma, en precursores musculares que inician el proceso de diferenciación terminal.

RESULTADOS

Los resultados presentados en esta sección fueron obtenidos para lograr gran parte de los objetivos de esta tesis de doctorado, previamente descritos.

El manuscrito fue enviado a una revista científica de corriente principal en el área de la biología celular.

NEDD4 REGULATES PAX7 LEVELS PROMOTING TERMINAL DIFFERENTIATION IN SKELETAL MUSCLE PRECURSORS.

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Keywords: Pax7, Nedd4, satellite cells, skeletal muscle, proteasome, ubiquitin.

ABSTRACT

The transcription factor Pax7 regulates skeletal muscle stem cell (satellite cells) fate by repressing the activity of the muscle regulatory factor MyoD. Hence, Pax7:MyoD protein ratios can determine maintenance of the committed-undifferentiated state or activation of the differentiation program. Pax7 protein concentration decreases sharply in differentiating myoblasts but is maintained in cells (re)acquiring quiescence, yet the mechanisms regulating Pax7 levels based on differentiation status are not well understood. Here we show that degradation of Pax7 requires Nedd4, making it a novel regulator of satellite cell fate. Our results indicate that Nedd4 is expressed in quiescent and activated satellite cells, that Nedd4 and Pax7 physically interact during early muscle differentiation, correlating with Pax7 ubiquitination and decline, and that Nedd4 loss of function prevents the differentiation-induced decrease in Pax7 protein. Moreover, Nedd4 ubiquitinates Pax7 directly, and even transient nuclear accumulation of Nedd4 induced both a drop in Pax7 levels and precocious muscle differentiation. Consequently, we propose a model in which temporal and spatial regulation of Nedd4 modulates the Pax7:MyoD ratio and therefore muscle progenitor cell fate.

INTRODUCTION

Skeletal muscle features an astounding capacity for regeneration in response to acute injury or disease. This response depends on a discrete population of tissue-specific adult stem cells known as satellite cells (SCs) (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011; Shi and Garry, 2006), which in undamaged muscle tissue are maintained in a quiescent state and are located between the plasma membrane and the basal lamina of the host muscle fiber (Mauro, 1961; Schultz et al., 1978). Upon muscle damage SCs become activated, proliferate, migrate and induce the expression of muscle regulatory transcription factor (MRF) MyoD. At this stage, SCs are committed to the myogenic lineage and are referred to as adult myoblasts. Eventually, adult myoblasts become further committed to terminal differentiation by expression of the MRF myogenin, withdraw from the cell cycle, and either fuse with existing fibers or fuse one to another to form new myofibers (Ciciliot and Schiaffino, 2010). The quiescent SC pool is replenished by self-renewal, ensuring subsequent muscle regeneration throughout adulthood (Chargé and Rudnicki, 2004).

Although the molecular regulation of muscle differentiation has been studied in detail, the mechanisms controlling SC maintenance and renewal remain to be elucidated. In this context, recent evidence underscores a critical requirement for Pax7 expression for SC specification (Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Seale et al., 2000) and function (Günther et al., 2013; Maltzahn et al., 2013). Through mechanisms not fully understood, Pax7 can repress MyoD transcriptional activity and terminal differentiation (Kumar et al., 2009; Olguín and Olwin, 2004; Olguín et al., 2007; Zammit, 2006). Accordingly, in the absence of Pax7 muscle progenitors undergo precocious differentiation *in vivo* (Günther et al., 2013; von Maltzahn et al., 2013). Interestingly, genetic and transcriptome analyses indicate that Pax7 alone can initiate the myogenic program, inducing MyoD and/or Myf-5 expression (Chen et al., 2006; Gros et al., 2005; Kassar-Duchossoy et al., 2005; McKinnell et al., 2007; Relaix, 2006; Relaix et al., 2005). These observations are consistent with a model where Pax7 can play dual roles in muscle progenitors (Olguín and Pisconti, 2012). Thus, differential regulation of Pax7 protein may be critical for SC function, as the Pax7:MyoD protein ratio affects myogenic progression (Olguín et al., 2007), while Pax7 rapidly declines upon myogenin induction (Olguin, 2011; Olguín and Olwin, 2004; Olguín et al., 2007; Zammit et al., 2004).

Since Pax7 function is crucial for SC fate, identifying mechanisms involved in Pax7 decline in differentiating cells and Pax7 retention in self-renewing SCs is expected to have profound implications for the understanding of SC biology.

The ubiquitin-proteasome system (UPS) mediates intracellular protein degradation in eukaryotic cells. This system regulates protein function and cell homeostasis with extreme substrate specificity and is therefore crucial for a variety of cellular processes (Hershko and Ciechanover, 1998). Accordingly, defects in UPS function lead to disease and cancer in humans (Schwartz and Ciechanover, 2009).

The UPS directs the covalent attachment of the 76aa protein ubiquitin to lysine residues of substrate proteins, targeting them for degradation by the 26S proteasome (Hershko and Ciechanover, 1998). Ubiquitin transfer occurs in a sequential, ATP-dependent reaction involving three different enzymes: E1 activating, E2 conjugating and E3 ligase; where the latter is critical to define the substrate specificity (Pickart, 2001).

Nedd4 (neural precursor cell-expressed developmentally down-regulated gene 4) is a member of the HECT (homologous to E6-AP carboxyl terminus) class of E3 ligases (Rotin and Kumar, 2009). They directly interact with substrate proteins through conserved WW domains and transfer ubiquitin from E2 via their HECT catalytic domain (Ingham et al., 2004). Nedd4 regulates membrane, cytoplasmic and nuclear proteins in a variety of cell contexts (Yang and Kumar, 2009), including skeletal muscle (Kumar et al., 1997), where participates in denervation and unloading-induced muscle atrophy (Koncarevic et al., 2007; Nagpal et al., 2012). Whereas Notch1 is the only Nedd4 muscle substrate reported to date (Koncarevic et al., 2007), Need4 is expressed in primary myoblast cultures; while activated SCs numbers in the gastrocnemius appear to increase upon denervation in muscle-specific Nedd4 knockout mice (Nagpal et al., 2012). Nevertheless, Nedd4 expression and function in SC remain to be determined.

Here we provide evidence indicating for the first time that Nedd4 can regulate muscle progenitor fate thought a mechanism involving control of Pax7 levels via the UPS, during early muscle differentiation.

RESULTS

Proteasome-dependent decrease in Pax7 levels during early differentiation of muscle progenitors.

We have previously shown that Pax7 declines concomitantly with the induction of myogenin expression in myoblasts cell lines; a process that was partially prevented by proteasome inhibition (Olguin, 2011; Olguín et al., 2007). Therefore, we asked whether Pax7 levels are regulated in a similar manner in activated SCs. For this, adult mouse primary myoblasts were allowed to differentiate in the presence or absence of the proteasome inhibitor MG132. As described earlier, Pax7 and myogenin exhibited a marked mutually exclusive expression pattern in vehicle-treated cells, as observed by indirect immunofluorescence (IF) (Fig. 1A, upper panel). However, MG132 treated cultures showed a significant increase (≥4 fold) in the percentage of Pax7(+)/myogenin(+) cells (Fig.1A, upper panel). Accordingly, Western blotting analyses revealed that Pax7 protein levels increased ≥ 2.5 fold upon MG132 treatment (Fig.1B). To further confirm the participation of the proteasome machinery, we compared changes in Pax7 levels upon incubation with two non-related proteasome inhibitors: MG132 and epoxomicin. As shown in Figure 1 (C-D), treatment with either inhibitor resulted in a dose-dependent increase of Pax7 protein in differentiating C2C12 myoblasts. Interestingly, this effect was consistently observed within a discrete window of time (~48 h after differentiation induction, Fig.1C). No changes in Pax7 levels were detected in cells maintained in proliferation conditions (Fig. 1C) or in cells analyzed later during differentiation (Fig. 1C), where any remaining Pax7 expression is largely confined to the "reserve population" of undifferentiated cells (Olguin and Olwin, 2004). On the other hand, Pax7 protein was detected only in nuclear extracts in both control and MG132 treated cells (Fig.1E), indicating that regulation of Pax7 levels occurs within this cellular compartment.

Pax7 is ubiquitinated in differentiating myogenic cells.

In an attempt to understand the mechanism(s) involved in Pax7 regulation, we used Tandem Affinity Purification (TAP) coupled to mass spectrometry in C3H10T1/2 cells co-expressing TAP-Pax7 and MyoD (Supplementary Material, Fig.E1A). Multidimensional Protein Identification Technology –MuDPIT– and *in vitro* pull-down assays revealed several

candidates Pax7-binding proteins, including members of the UPS (Fig.E1B). Based on these findings and our previous results, we looked for potential roles for this particular set of putative interactions during the post-translational control of Pax7.

First we verified *in vivo* Pax7 ubiquitination in C2C12 cells by bimolecular fluorescence complementation assays (BiFC) (Fang and Kerppola, 2004). Live-cell fluorescent signal was only reconstituted when Pax7-[Venus C-terminus] fusion protein (Pax7-VC) and ubiquitin-[Venus N-terminus] fusion protein (ubiquitin-VN) were co-expressed (Fig. 2A; Fig.E2). Importantly, complementation was detected in the cell nucleus and enhanced by MG132 treatment, correlating with increased Pax7-VC levels (Fig.E1A). Control complementation mediated by interacting proteins bFos-VC and bJun-VN remained unaffected by the presence of MG132 (Fig. 2A), as previously described (Fang and Kerppola, 2004). Ubiquitin-Pax7 complexes were also detected by denaturating Ni-NTA affinity purification of total ubiquitinated proteins from C2C12 myoblasts expressing myc-6xHis-Ub (Fig. 3B), suggesting that Pax7 is in fact ubiquitinated. Unexpectedly however, ubiquitin-Pax7 appeared mainly as discrete band(s) instead of the ladder/smear-like signal characteristic of poly-ubiquitinated proteins targeted for proteasome-dependent degradation (Fig. 3B, compare IP vs. input, Fig. 3B). Furthermore, equivalent ubiquitin-Pax7 complexes were obtained *in vitro* upon incubation of purified GST-Pax7 fusion protein with a UPS-enriched HeLa cell extract plus ATP (Fig. 3C). In this scenario, we considered two alternative explanations: i) Pax7 was modified by addition of single ubiquitin molecule(s) (i.e. mono-ubiquitination or multi-mono-ubiquitination) or ii) Pax7 co-immunoprecipitation was indirect and due to its interaction with different ubiquitinated proteins. The later argument seemed less likely due to the denaturating conditions used during Ni-NTA affinity purification and by the observation that equivalent ubiquitin-Pax7 species were obtained *in vivo* and *in vitro*. In order to test the remaining possibility, *in vivo* ubiquitination assays were performed in C3H10T1/2 cells co-expressing Pax7 and either HA-tagged ubiquitin or HA-tagged mutant ubiquitin proteins lacking either a critical lysine residue for lysine 48-based polyubiquitin chain formation (K48R) or all lysines (K0). Both mutants prevent polyubiquitin chain formation while still allowing ubiquitin conjugation to the target protein (Ben-Saadon et al., 2006; Carvallo et al., 2010; Chau et al.,

1989). Ubiquitin-Pax7 complexes with equivalent relative molecular mass were detected in all three conditions after denaturating IP of Pax7 (Fig. 3D).

Together, these results support the concept that the UPS regulates Pax7 protein levels in differentiating myogenic cells. In this context, we have previously observed that deletion of Pax7 C-terminus domain (Pax7-ΔC) results in significantly higher expression when compared to the full-length Pax7 protein (Olguín et al., 2007). Accordingly, incubation with MG132 results in Pax7, ΔN and ΔHD Pax7-deletion-mutant accumulation when ectopically expressed in C3H10T1/2 cells. This accumulation is not observed for Pax7-ΔC (Fig. 3E), suggesting that Pax7 C-terminus domain is required (directly or indirectly) for Pax7 UPS-mediated regulation.

Ubiquitin ligase Nedd4 is a novel regulator of Pax7 stability in muscle progenitors.

Among the candidate Pax7 regulators uncovered in our proteomics analysis we identified Nedd4 (Fig. E1B), a member of the HECT superfamily of E3 ligases (Yang and Kumar, 2009), whose role in adult muscle progenitors had not been described. Therefore, we first examined its expression in quiescent and activated SCs. Nedd4 protein was detected in a subset of cells located underneath the basal lamina from *tibialis anterior* muscle sections (Fig. 3A, upper left panel). The identity of these cells was further confirmed by co-localization with the SC marker Pax7 (Fig. 3A, upper right panel). Although there was almost complete Pax7 and Nedd4 co-expression, Nedd4 was also expressed in Pax7(-) interstitial cells (Fig. 3A, asterisk). This is not unexpected, since Nedd4 has been described in a variety of cell types. Additionally, low Nedd4 signal was observed at the subsarcolemmal region in skeletal muscle sections, consistent with previous expression studies (Koncarevic et al., 2007).

We also examined Nedd4 expression during *ex-vivo* SC activation and proliferation in isolated myofibers cultures (Fig. 3A lower panels; 0.5 and 72 h, respectively). Interestingly, Nedd4 exhibited a marked cytoplasmic expression pattern both in quiescent and activated SCs. This subcellular distribution was also observed in differentiating C2C12 myoblasts (Fig. 3B).

Next, we investigated the nature of the interaction between Pax7 and Nedd4 by *in vitro* GST pull-down followed by Western blotting. Nedd4 was significantly enriched when using GST-Pax7 as bait, while no interaction with GST was observed (Fig. 3C). Similar results were obtained performing pull-down using *in vitro* translated [^{35}S]-Nedd4 (Fig. E1B), indicating

that Pax7 and Nedd4 can physically interact. This interaction also appears to occur *in vivo*, since both proteins were co-IP from C2C12 myoblasts whole-cell extracts (Fig. 3D). Notably, co-IP was observed upon induction of differentiation (Fig. 3D) but not in cells maintained in proliferating conditions, consistent with our previous observations (see Fig. 1).

Since Nedd4 regulates the stability of several ligand proteins (Yang and Kumar, 2009), we hypothesized that Nedd4 knockdown would result in increased Pax7 protein levels. We tested this idea using the C3H10T1/2 myogenic conversion model, since these cells express endogenous Nedd4 but not Pax7, thus allowing co-transfection of Pax7 cDNA and Nedd4 siRNA in order to evaluate the effect on Pax7 levels by Western blotting. Accordingly, Nedd4 knockdown (~52%) results in a >8 fold increase in Pax7 protein, while no significant changes were observed upon co-expression of a non-targeting siRNA (Fig. 3E). Next, we asked if Nedd4 could directly ubiquitinate Pax7 *in vitro*, using immobilized GST-Nedd4 protein in order to facilitate separation of ubiquitinated Pax7 from Nedd4-ubiquitin products (see Materials and Methods). Under these conditions, we detected ubiquitinated species that were absent when either Pax7 or ATP was omitted from the reaction (Fig. 3F; arrow heads at >55-72 KDa, left panel). Importantly, these products do not correspond to immobilized Nedd4 or unmodified Pax7 (Fig. 3F), since no products were detected when Nedd4 was replaced in the reaction by immobilized GST-only protein (Fig. 3F, right panel).
Taken together, these observations indicate that i) Nedd4 and Pax7 are co-expressed and physically interact in adult myoblasts, and ii) Nedd4 can directly modify Pax7 protein.

Nedd4 nuclear import/export is critical for regulation of Pax7 protein.

Intriguingly, Pax7 and Nedd4 appeared to be localized at different subcellular compartments, which would prevent their interaction *in vivo*. However, previous studies showed that Nedd4 possesses functional nuclear localization and nuclear export signals (Hamilton et al., 2001). The latter appears to be particularly strong, limiting Nedd4 presence in the nucleus to almost undetectable levels. This effect can be prevented by inhibiting CRM1 (chromosome region maintenance 1)/exportin 1-dependent export (Kudo, 1998), which results in nuclear accumulation of Nedd4 in HeLa cells (Hamilton et al., 2001). Therefore, we decided to determine if nuclear translocation of Nedd4 would allow interaction with Pax7 in myogenic cells. For this, co-IP experiments were performed in isolated cytoplasmic and nuclear fractions

of C2C12 myoblasts. As described previously, Pax7 protein was only detectable in the nuclear compartment (Fig. 1D). Although highly enriched in cytoplasmic fractions, lower levels of Nedd4 (26 ± 10%) were detected in C2C12 nuclear extracts (Fig. 4A). Remarkably however, Nedd4 was efficiently co-immunoprecipitated from nuclear fractions using an anti-Pax7 antibody (Fig. 4B), suggesting that at least some Nedd4 protein was imported to the nucleus and was interacting with Pax7. To further test this idea, C2C12 cells were treated with the CRM1 inhibitor leptomycin B (LMB). In line with previous reports, LMB resulted in a significant increase in Nedd4 in myoblast nuclei, as determined by confocal microscopy and Western blotting (Fig. 4C). Interestingly, nuclear accumulation of Nedd4 correlated with a significant decrease in the Pax7 signal (Fig. 4C), which was further confirmed by Western blotting (≥50% decrease compared to vehicle, Fig. 4D). Importantly, Pax7 decrease upon LMB treatment was efficiently prevented by proteasome inhibition (Fig. 4E), indicating that LMB-induced changes in Pax7 levels require active UPS.

We have previously proposed that regulation of the Pax7:MRFs ratio is critical to maintain muscle progenitors in a self-renewing/proliferating state ($Pax7^{high}/MRF^{low}$) or allow their commitment to terminal differentiation ($Pax7^{low}/MRF^{high}$). In this context, we hypothesized that a decrease in Pax7 levels upon LMB treatment would result in precocious differentiation commitment. To test this possibility, LMB was removed after a 6 h treatment, and the cells were maintained in culture for an additional 48 h prior to fixation. Under these conditions, LMB-induced nuclear accumulation of Nedd4 was effectively reversed upon removal, as determined by loss of Nedd4 nuclear signal observed by IF (Fig. 5A). Nevertheless, Pax7 levels remained below control after transient LMB treatment (Fig. 5B), which correlated with a >2 fold increase in the percentage of myogenin (+) cells (Fig. 5B) and an increase in the population of cells expressing myosin heavy chain (Fig. E4), indicating progression towards terminal differentiation. A significant increase in myogenin (+) cells was also observed in LMB-treated cells maintained in proliferating culture conditions, suggesting that a transient decrease in Pax7 protein levels is sufficient to allow commitment to differentiation. Since Nedd4 is one of many proteins exported by a CRM1/exportin-1 dependent mechanism (Fig. E3A), we confirmed the specific requirement for Nedd4 by evaluating the effect of LMB on

cells previously transfected with a Nedd4 siRNA or a non-targeting control siRNA: Nedd4 knockdown blocked the LMB-induced drop in Pax7 levels by at least 50% (Fig. 5C).

Together, these results suggest that regulation of Nedd4 sub-cellular localization is critical to modulate Pax7 protein levels and muscle progenitor fate.

DISCUSSION

Adult muscle progenitor fate is influenced by a functional interaction between the transcription factor Pax7 and members of the MyoD family of MRFs, which is thought to permit them to acquire lineage identity while preventing commitment to terminal differentiation (Olguín and Pisconti, 2012). We have hypothesized that changes in the Pax7:MyoD protein ratio may act as a molecular rheostat to fine-tune the timing of these events. In this scenario, post-translational modifications can play an important role in controlling Pax7 expression and function in a context dependent manner (Kawabe et al., 2012; Luan et al., 2012; Olguin, 2011). In the present study we describe a novel mechanism by which the E3 ubiquitin ligase Nedd4 connects the UPS and the regulation of Pax7 levels in cells undergoing muscle differentiation.

Nedd4 deletion leads to lethality before birth (Cao et al., 2008; Fouladkou et al., 2010; Liu et al., 2009). Interestingly, skeletal muscle and neuromuscular junction development in Nedd4-null mice are impaired, indicating a possible role for Nedd4 in muscle formation during embryogenesis (Cao et al., 2008; Fouladkou et al., 2010; Liu et al., 2009). In adult skeletal muscle, Nedd4 expression increases upon both denervation and unloading-induced atrophy, while muscle-specific Nedd4 knockout mice retain muscle mass after denervation (Koncarevic et al., 2007; Nagpal et al., 2012); suggesting that Nedd4 directly participates in the development of the atrophic phenotype under very specific conditions, which in turn implies that different pathways in the muscle cell can lead to atrophy. Despite these findings, potential Nedd4 function(s) in muscle maintenance and repair have not been described. In this scenario, our results show for the first time that i) Nedd4 is expressed in quiescent and activated SCs and ii) that Nedd4 function regulates adult muscle progenitor cell fate by affecting Pax7 levels.

Pax7 levels are controlled by proteasomal activity.

Pax7 levels rapidly decline as myogenin expression increases, leading to a mutually exclusive expression pattern in muscle progenitors. Proteasome inhibition partially recovered Pax7 expression in myogenin(+) cells, suggesting for the first time that Pax7 levels were post-translationaly regulated (Olguín et al., 2007). Here we showed that proteasome inhibition in

primary adult myoblasts also leads to Pax7 retention in myogenin(+) cells (Fig. 1), strongly suggesting that proteasome-dependent degradation of Pax7 is a physiologically relevant mechanism to control the Pax7:MRFs balance. Interestingly, the timing of Pax7 decline is under fine control, since Pax7 accumulation upon proteasome inhibition was restricted to early differentiation in C2C12 myoblasts (Fig. 1).

Pax3 -a close Pax7 homolog- is also subject to UPS regulation (Boutet et al., 2007). In their study, Boutet and colleagues not only show that Pax3 is marked for degradation via a non-canonical pathway but also ruled out a similar regulation for Pax7. This conclusion is based on two observations: i) when Pax3 dropped, Pax7 levels were unaffected and ii) site-directed mutagenesis introducing a lysine in position 475, which is key for Pax3 degradation, turned Pax7 susceptible to UPS-mediated degradation. The apparent contradiction with the results presented here may highlight key functional differences between the two Pax proteins. First, in the referenced study, Pax3 levels increased upon proteasome inhibition in proliferating C2C12 and primary myoblasts while Pax7 remained unchanged. As shown in Fig. 1C, Pax7 levels also remain unaffected by MG132 treatment in myoblasts maintained in proliferating culture conditions. Nevertheless, Pax7 levels are clearly affected by proteasome inhibition in differentiating myoblast cultures. Upon myogenic progression, Pax7 expression is maintained in a small population of undifferentiated "reserve cells" (Yoshida et al., 1998). Interestingly, proteasome inhibition in differentiated cell cultures has no effect on the remaining Pax7 levels, supporting the idea that the UPS regulates Pax7 in a cell context-dependent manner. Together, these observations are compatible with a step-wise decline of Pax3 followed by Pax7 during myogenic progression. Differences in the nature and regulation of the enzymes and signaling pathways involved in Pax3 and Pax7 degradation can also explain these disparities.

Pax7 mono-ubiquitination as signal for proteasomal degradation?

Using at least three different approaches (i.e. BiFC, immunoprecipitation and *in vitro* ubiquitination), we determined that Pax7 is ubiquitinated during muscle differentiation (Fig. 2). Interestingly, Pax7 appears to be mono-ubiquitinated (Fig. 2B-E) in muscle progenitors as shown previously for Pax3 (Boutet et al., 2007). Importantly, Pax7 mono-ubiquitination is catalyzed by Nedd4 as shown in vitro (Fig. 3F). Despite of this, we were not able to identify in the present study a specific Pax7-residue subject to this modification. However, studies using

deletion mutants suggested Pax7 carboxy terminus might be directly or indirectly involved in this process (Fig. 2F). Thus, it will be interesting to study the functional relevance of Pax7 mono-ubiquitination in its activity and degradation.

Ubiquitinated protein recognition by the proteasome is classically attributed to the presence of polyubiquitin chains based in K48 linkages between ubiquitin monomers (Chau et al., 1989), which are specifically recognized by the ubiquitin receptor subunits of proteasome complex Rpn10/S5a and Rpn13/ARM1 (Deveraux et al., 1994; Husnjak et al., 2008). However, non-canonical ubiquitin signals have also been described (Ciechanover et al., 1999; Finley, 2009; Kravtsova-Ivantsiv and Ciechanover, 2012; McDowell and Philpott, 2013). In this context, an increasing number of examples support the role of mono-ubiquitin modification as a *bona fide* signal for protein degradation (Boutet et al., 2007; Kravtsova-Ivantsiv et al., 2009; Yin et al., 2010), while the detailed mechanism remains unclear. Targeting mono-ubiquitinated proteins for degradation involves ubiquitin receptors carrying modified substrates to the proteasome (Finley, 2009; Boutet et al., 2007). In this context, remains to be determined if Pax7 UPS-dependent regulation involves a similar mechanism.

Regulation of Nedd4 function in adult muscle precursors.

Here we show that Nedd4 E3 ligase interacts with and regulates Pax7 protein levels in muscle precursors (Fig. 3). Nedd4 interaction with its substrates requires ubiquitin interacting motifs and the presence of ubiquitin binding domains in target proteins subjected to mono-ubiquitination (Polo et al., 2002; Woelk et al., 2006). Additionally, Nedd4 catalytic activity is regulated in *cis* via intramolecular interaction of the C2 and HECT domain, in which C2 domain inhibits Nedd4 function (Wiesner et al., 2007). Identification of the detailed interaction surface between Nedd4 and Pax7 may be important to understand molecular determinants of Pax7 ubiquitination.

Nedd4 activity can be regulated via extrinsic stimuli. For example, tyrosine phosphorylation mediated by Src kinases in response to FGFR activation antagonizes C2 domain mediated auto-inhibition to activate its ubiquitin-ligase activity (Persaud et al., 2014). Thus, complete mechanisms connecting SC niche signaling with Nedd4 activity are still missing and could be relevant to understand Pax7 regulation in quiescent and activated SCs. In this line, a critical

subject is the relationship between post-translational and post-transcriptional regulation of Pax7, since microRNA-dependent Pax7 decrease (via miR-1 and miR-206) has been described during the proliferation-to-differentiation transition in primary mouse myoblasts (Chen et al., 2010). Accordingly, hypoxic conditions favor quiescence/self-renewal in proliferating myoblasts by up regulating Pax7 expression, via a mechanism involving Notch-dependent repression of miR-1 and miR-206 expression (Lui et al., 2012).

We provided evidence indicating that Nedd4 shuttles between the cytoplasm and the nucleus in a myogenic context (Fig. 4). Transient disruption of Nedd4 nuclear export (i.e. pharmacologically induced nuclear accumulation of Nedd4) is sufficient to decrease Pax7 levels (Fig. 4-5). Most significantly, this nuclear Nedd4-dependent Pax7 decline results in precocious commitment to differentiation (Fig. 5). Thus, Nedd4 nuclear import/export appears to be a rate-limiting step and subject to specific regulation in myogenic cells, since Nedd4 over-expression in C2C12 or C3H10T1/2 cells co-expressing Pax7 results in accumulation of Nedd4 at the cytoplasm, while no significant changes in Pax7 levels were observed prior to differentiation induction (Bustos and Olguin, unpublished observations).

Physiological relevance of UPS/Nedd4 mediated Pax7 regulation.

SCs-specific Pax7 knockout mouse models show dramatic loss of muscle regenerative capacity upon injury due to progressive failure in proliferation and self renewal capacities (Günther et al., 2013; von Maltzahn et al., 2013). Furthermore, recent findings showing that persistent Pax7 expression may underlie SC dysfunction during cancer cachexia (He et al., 2013), indicate that deregulation of Pax7 levels may play a role in diseased muscle.
UPS dependent regulation appears to be critical for stem cell function (Heuzé et al., 2008; Moran-Crusio et al., 2012; Naujokat, 2009; Naujokat and Šarić, 2007; Reavie et al., 2010; Tuoc and Stoykova, 2010), including muscle precursor differentiation (Gardrat et al., 1999; Kim et al., 1998; Mugita et al., 1999). In this scenario, ubiquitination could function to connect extrinsic and/or intrinsic pathways to regulate the Pax7:MyoD ratio, and thus, influence muscle progenitor cell fate. We believe our findings are relevant not only to understand how Pax7 protein levels are regulated during muscle cell differentiation, but also to establish UPS and Nedd4 as important determinants of SCs function and muscle tissue maintenance.

MATERIALS AND METHODS

Tandem Affinity Purification (TAP)/ mass spectrometry.

C3H10T1/2 cells were transduced with Adenoviral vectors for tandem affinity purification (TAP) tagged-Pax7 and MyoD in a 1:1 Pax7-to-MyoD ratio . At this ratio myogenic conversion is significantly reduced (~50%) (Olguín and Olwin, 2004) . Cells were then treated with MG132 in order to inhibit degradation of multiple cellular proteins and to facilitate the identification of positive and negative Pax7 regulators (Fig. E1A).InterPlay Adenoviral TAP System (Agilent Technologies, Santa Clara, CA, USA) was used for TAP-tagged Pax7 purification. TAP eluates were analyzed through Multidimensional Protein Identification Technology (MuDPIT) as described (Washburn et al., 2001) and compared to eluted fractions from cells expressing TAP-tagged chloramphenicol acetyltransferase (TAP-CAT). MuDPIT data was analyzed and validated as described (Cociorva et al., 2007). The complete data set is available at http://fields.scripps.edu/publicworks.php.

Cell culture.

Adult mouse primary myoblasts (pMbs) and isolated myofibers were obtained as previously described (Olguín and Olwin, 2004) and cultured in F12-C (Life technologies, Carlsbad, CA, USA) supplemented with 15% horse serum (HS) (Hyclone, South Logan, UT, USA) and 1 nM FGF-2 (growth medium) and maintained at 37ºC with 5% CO_2. For differentiation assays, myoblasts were cultured in differentiation medium (F12-C with 7.5% HS) and isolated myofibers in growth medium without FGF-2.

C2C12 and C3H10T1/2 cell lines were maintained in DMEM (Life technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA) at 37ºC with 5% CO_2. For differentiation assays C2C12 myoblasts were plated at 8000 cells/cm^2 24 h prior to differentiation induction (DMEM 5% HS). 48 h post differentiation induction, C2C12 cells were supplemented with 0.1 nM 1-β-D-arabinofuranosylcytosine (AraC) (EMD Millipore, Billerica, MA, USA). For ubiquitination assays, C3H10T1/2 cells were subjected to myogenic conversion via MyoD ectopic expression, as described (Olguín and Olwin, 2004). When required, cells were incubated with 0.5-25 μM MG132, 1-5 μM epoxomicin or DMSO (all from Sigma-Aldrich, St. Louis, MO,USA) for 6 h prior to lysis or fixation. To block

CRM-1/exportin-1, cells were treated with 30 nm Leptomycin-B (Sigma-Aldrich, St. Louis, MO, USA) or vehicle for the indicated periods prior to fixation or lysis.

Subcellular fractionation.

C2C12 cells were collected in cold PBS and resuspended in buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM DTT) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO,USA) and incubated 30 min on ice. Cells were passed 40 times through a 25G x 5/8 needle, and centrifuged at 700 g for 5 min at 4ºC; supernatant was stored as cytoplasmic fraction. Pelleted fraction was then washed three times in buffer A, resuspended in buffer B (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT and 25% glycerol), incubated on ice for 20 min and centrifuged 2 min at 14.000 rpm at 4ºC to obtain the nuclear fraction.

Plasmid and siRNA transfection.

pRSV-MyoD, pCDNA3-Pax7d, pCDNA3-myc-NLSPax7FL, pCDNA3-myc-NLSPax7ΔC, pCDNA3-myc-NLSPax7ΔN, pCDNA3-myc-NLSPax7ΔHD plasmids were described previously (Olguín et al., 2007). pCS2-UbHA, pCAGGS-UbHA-WT, pCAGGS-UbHA-K48R, pCAGGS-UbHA-K0 and gap43-mRFP plasmids were donated by Dr. Juan Larraín (Santiago, Chile). pCMV7-myc-6xHis-Ub was donated by Dr. Kristen Bartel (Boulder CO, USA) EYFP-MEM plasmid was obtained from Clontech (Clontech, Mountain View, CA, USA). pBiFC-bFos-VC155, and pBiFC-bJun-VN155(I152L) were previously described (Kodama and Hu, 2010) and were obtained from Dr. Chang-Deng Hu, (West Lafayette, IN, USA). pBiFC-Pax7-VC155 was generated by PCR cloning of the specific cDNA from pCDNA3-Pax7d. Primers used were FW: 5'-CCGGAATTCGGATGGCGGCCCTTCCC-3', RV: 5'-CCGCTCGAGAGTAGGCTTGTCCCGTTTCCA-3'. pBiFC-Ub-VN155(I152L) was generated by PCR cloning of HA-ubiquitin cDNA from pCS2-UbHA plasmid. Primers used: FW: 5'-CCGGAATTCATGCAGATCTTCG-3' RV: 5'-CCGCTCGAGCCCACCTCTCAGACG-3' . EcoRI and XhoI restriction sites were generated by PCR, for directional product insertion in pBiFC-VC155 and pBiFC-VN155(I152L) backbones. Fusion protein generation and Pax7-VC155 and Ub-VN155(I152L) expression was confirmed via Western blot.

For knockdown, a pool of Nedd4 siRNAs (QIAGEN, Valencia, CA, USA) and siCONTROL RISC-free siRNA (Dharmacon, Lafayette, CO, USA) were used. Expression vectors and siRNAs were transfected using Lipofectamine 2000 (Life technologies, Carlsbad, CA, USA) or Superfect (QIAGEN, Valencia, CA, USA) according the manufacturer's instructions.

Western blotting and immunoprecipitation.

Cells were lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% IGEPAL, protease and phosphatase inhibitors) and 10-30 μg of total protein were loaded into 10% SDS-PAGE gels. Proteins were transferred to a PVDF membrane and probed with the following primary antibodies and dilutions: mouse monoclonal anti-Pax7, 1:10; mouse monoclonal (F5D) anti-myogenin (both from Developmental Studies Hybridoma Bank, Iowa City, IA, USA), 1:5; mouse monoclonal anti-tubulin, 1:10000; mouse monoclonal anti-HA HRP conjugated, 1:4000 (both from Sigma-Aldrich, St. Louis, MO,USA); mouse monoclonal anti-HDAC2 [3F3], 1:5000; rabbit polyclonal ChiP-grade anti-HA tag, 1:10000; rabbit polyclonal anti-Nedd4, 1:10000 ; rabbit monoclonal anti-GFP E385, 1:5000 (all from Abcam, Cambridge, CAM, UK); mouse monoclonal anti-GAPDH (EMD Millipore, Billerica, MA, USA), 1:10000; mouse monoclonal 9B11 myc-tag (Cell Signaling, Danvers, MA, USA), 1:1000; rabbit polyclonal anti-GST (kind gift of Dr. María Paz Marzolo, Santiago, Chile), 1:5000 and mouse monoclonal P4D1 anti-ubiquitin (Santa Cruz Biotechnology, Dallas, TX, USA), 1:500. As secondary antibodies HRP conjugated anti-mouse IgG and anti-rabbit IgG (Cell Signaling, Danvers, MA, USA) were used at 1:5000. HRP activity was detected using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Rockford, IL, USA). For cell based co-immunoprecipitation (co-IP) assays, C2C12 cell were lysed in RIPA or C2C12 fractions were obtained in buffer A (cytoplasmic) or buffer B (nuclear) and subjected to an IP protocol as described (Olguín et al., 2007).

Immunofluorescence.

Cells were fixed in 4% paraformaldehide (PFA) for 20 min and subjected to standard indirect immunofluorescence (Olguín et al., 2007). Primary antibodies and dilutions were as following: mouse monoclonal anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), 1:5; rabbit polyclonal anti-myogenin M-225 (Santa Cruz Biotechnology, Dallas, TX, USA),

1:200; chicken anti-Syndecan-4 (Cornelison et al., 2004) 1:500; rabbit polyclonal anti-Nedd4 (Abcam, Cambridge, CAM, UK), 1:1000. Secondary antibodies and dilutions were: goat anti-mouse Alexa 594, 1:500; goat anti-rabbit Alexa 488, 1:500; goat anti-mouse Alexa488, 1:500 (all from Life technologies, Carlsbad, CA, USA) and donkey anti-chicken-AMCA (Jackson IR, West Groove, PA, USA), 1:500. Vectashield (Vector labs, Burlingame, CA, USA) was used for mounting. Images were acquired using an IX71 microscope (Olympus, Center Valley, PA, USA) equipped with a QICam FAST QImaging camera or an Eclipse C2 spectral imaging confocal microscope (Nikon, Tokyo, Japan).

Muscle section staining.

Tibialis anterior muscles from 2-3 month-old female B6D2F1/J or C57BL/6J mice were dissected and snap-frozen in liquid nitrogen-chilled isopentane. Frozen muscles were then cryosectioned at 10 μm thickness and fixed in 4% PFA/PBS. Sections were permeabilized in 0.5% Triton X-100/PBS, blocked with 3% BSA/PBS and incubated with rat monoclonal anti-Laminin (LN) antibody (Sigma-Aldrich, St. Louis, MO,USA) at 1:2000 and rabbit polyclonal anti-Nedd4 antibody (Abcam, Cambridge, CAM, UK) at 1:1000 diluted in blocking buffer. Alternately, sections were permeabilized in Triton buffer (0.5% Triton X-100, 20 mM Tris-HCl pH: 7.4, 100 mM NaCl, 0.2% sodium azide) and then washed with Tris-glycine Buffer (25 mM Tris-HCl pH 7.4, 200 mM glycine, 0.2% Sodium Azide, 100 mM NaCl). Sections were blocked in 10% normal goat serum with 1% IGEPAL and incubated with mouse monoclonal anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), 1:5 and rabbit polyclonal anti-Nedd4 (Abcam, Cambridge, CAM, UK), 1:1000 in blocking serum. Sections were washed with Tween-20 Buffer (2.5 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) and then fixed with 4% PFA/PBS and washed extensively with Tween-20 Buffer. Donkey anti-rabbit IgG Alexa-555, goat anti-mouse Alexa 594 and donkey anti-rat IgG Alexa-488 (all from Life technologies, Carlsbad, CA, USA) were used as secondary antibodies at 1:500 dilution. Slides were mounted in Vectashield with DAPI (Vector labs, Burlingame, CA, USA), and imaged for analysis using a BX61 (Olympus, Center Valley, PA, USA) or a Diaphot Eclipse E600 microscope (Nikon, Tokyo, Japan).

Ubiquitin-mediated fluorescence complementation.

General protocol was performed as described previously (Fang and Kerppola, 2004; Kodama and Hu, 2010) with minor modifications. Briefly, C2C12 cells were transfected with plasmids encoding C-terminus fragment of Venus Fluorescent protein (VC155) fused to Pax7 -or bFos- and N-terminus part of Venus (VN155-I152L) fused to ubiquitin -or bJun- as specified. Gap43-mRFP was included as a transfection marker. 24 h post transfection, Venus and mRFP fluorescence were determined in living cells, using an IX71 inverted fluorescence microscope (Olympus, Center Valley, PA, USA) equipped with a QICam FAST QImaging digital camera. When indicated, 10 μM MG132 or DMSO was added 6 h prior to examination.

Cell-based ubiquitination.

C2C12 cells were transfected with Pax7 and myc-6xHis-ubiquitin, induced to differentiate for 48 h, incubated with MG132 6 h prior to lysis and subjected to a His-ubiquitin based assay as described (Jin et al., 2012) with minor modifications. Cells were treated with 25 μM MG132 for 6 hours and then scrapped in PBS. 10 % of cells were stored as inputs. Cells were pelleted for 10 minutes at 1000 rpm and resuspended in buffer A2 (6 M guanidine chloride, 0.1 M Na_2HPO_4/NaH_2PO_4 and 10 mM immidazole, pH 8.0) and incubated with 50 μl of Ni-NTA agarose (QIAGEN, Valencia, CA, USA) for 3 h at room temperature. After binding, Ni-NTA beads were washed twice with buffer A2, twice with buffer A2/TI (1 volume of buffer A2 and 3 volumes of buffer TI) and once with buffer TI (25 mM Tris-HCl, 20 mM imidazole, pH 6.8). Then proteins were eluted with buffer ETI (25 mM Tris-HCl, 300 mM imidazole, pH 6.8) for 10 minutes at room temperature. Beads were removed by centrifugation and eluted proteins were analyzed by SDS-PAGE and western blotting. Alternatively, C3H10T1/2 cells were transfected with HA-tagged ubiquitin, Pax7 or MyoD48 h post transfection cells were incubated with MG132 6 h prior to lysis scrapped and subjected to a denaturing IP. Cell pellet was boiled 5 min at 100º C in denaturing buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% SDS, 10 mM DTT, 10 mM iodoacetamide, 5 mM N-ethylmaleimide and protease inhibitors). Samples were then diluted 1:10 in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM iodoacetamide, 5 mM N-ethylmaleimide and protease inhibitors) and extracted for 30 min at 4ºC. Samples were then subjected to a standard IP

protocol using anti-Pax7 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) at 1:10. Western Blot was performed using anti-HA tag (Abcam, Cambridge, CAM, UK), 1:10000 or anti-HA HRP conjugated (Sigma-Aldrich, St. Louis, MO,USA), 1:4000 antibodies.

GST pull-down.

5 µg of GST or GST-Pax7 were incubated with 0.25 µg of Nedd4 in GST pull-down buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100 and protease inhibitors) for 20 h at 4ºC and then incubated with 10% v/v glutathione-agarose beads (Thermo Fisher Scientific, Rockford, IL, USA) 2 h at 4ºC. Beads were then washed with GST pull-down buffer supplemented with 300 mM NaCl and proteins were eluted with SDS-PAGE sample buffer and boiled prior to Western blot analysis. For candidate interaction studies, [^{35}S]-labeled proteins were obtained by coupled *in vitro* translation in rabbit reticulocyte extracts (Promega, Madison, WI, USA) as described (Olguín et al., 2007) and pulled-down with GST or GST-Pax7.

***In vitro* ubiquitination.**

1µg of purified GST-Nedd4 or GST-only were pre-bound to 50 ul glutathione-agarose beads (Thermo Scientific, Rockford, IL, USA) for 2 h at 4ºC in GST pull-down buffer. Immobilized GST-Nedd4 was incubated with 5 µg of purified Pax7 protein in a 100 µl reaction containing: 10 µg of recombinant HA-Ubiquitin, 200 ng of Ubiquitin Activating Enzyme UBE1 (E1), 300 ng of UbcH5b (E2), 1X Energy regeneration solution (ERS) (all from Boston Biochem, Cambridge, MA, USA), and 50 mM HEPES, pH 7,5 buffer. Reactions were incubated at 30 ºC for 1 h and then centrifuged to separate immobilized GST-Nedd4 from supernatants. Supernatants containing ubiquitination reaction outcomes were analyzed by SDS-PAGE and Western blotting. Nedd4 presence in the assay was confirmed by analyzing the gluthatione-agarose beads by SDS-PAGE and Western blotting (Supplementary Fig. E3B). E3 activity was determined by detection of auto-ubiquitinated Nedd4 in parallel assays (data not shown), as described previously (Woelk et al., 2006).

Alternatively 5 µg of GST-Pax7 or GST-only proteins were incubated in a 50 µl reaction with S-100 HeLa cell fraction 3.7 mg/ml as UPS source, supplemented with 5 µg of HA-ubiquitin,

1X ERS and 8 µM MG132 and 250 ng/µl ubiquitin aldehyde (all from Boston Biochem, Cambridge, MA, USA). Reactions were incubated at 30ºC for 1 h and diluted in 500 µl of GST pull-down buffer. Samples were then subjected to GST pull-down and Western blotting.

Data analysis.

Data is presented as representative experiments and quantifications are shown as mean ± standard error of the mean (s.e.m.) of three biological replicates. For immunofluorescence analysis at least 1000 cells -from three experimental replicates- were analyzed per treatment, unless indicated. For Western blots, densitometry analyses were performed using ImageJ software (NIH). Data was then plotted in Excel (Microsoft, Redmond, WA, USA) or GraphPad Prism software (GraphPad software Inc. La Jolla, CA, USA). Student's t-test or one-way ANOVA followed by Tukey´s post hoc test was performed to determine statistical significance. Immunofluorescence images were processed using Photoshop software (Adobe, San Jose, CA, USA) and final figures were assembled using Illustrator software (Adobe, San Jose, CA, USA).

Ethical issues.

All mice experiments were performed according to National Commission for Science and Technology (CONICYT) guidelines and approved by Facultad de Ciencias Biológicas of P. Universidad Católica de Chile bioethics and biosecurity committee.

ACKNOWLEDGMENTS

We thank Dr. Juan Larrain for Ub-HA, UbK48R, UbK0 and gap43mRFP plasmids, Dr. Sharad Kumar for mNedd4 and Dr. Chang-Deng Hu for VC155 and VN155(I152L) constructs. We are indebt to Dr. Enrique Brandan, Dr. Felipe Court and Dr. María Paz Marzolo for reagents and access to indispensable equipment. We greatly appreciate Dr. Patricia Burgos, Pamela Farfán and Dane Lund for troubleshooting with us UPS related protocols. Finally, we thank all members from the Olguín lab for valuable discussions and suggestions.

AUTHOR CONTRIBUTIONS

FB and HO conceived and designed the experiments; FB, EdV, JT and HO performed the experiments; FB, EdV, JT and HO analyzed the data; DC, BO, JY and HO contributed reagents/equipment/analysis tools and data analysis; FB, EdV, and HO wrote the paper.

FUNDING

This work was supported by the National Fund for Scientific and Technological Development (FONDECYT) [grant number 1130631] to HO; International Research Visit Fellowship from Fulbright Commission to FB; National Doctoral Fellowship, Doctoral Research Visit Fellowship “Becas-Chile” and Doctoral Thesis Support Fellowship AT- 24121135 from CONICYT to FB; Institutional Fellowship for graduate students (VRI-PUC) to EdV. Funding for DDWC has been provided by NIH AR062836. Funding for JRY has been provided by National Institutes of Health (NIH) grants P41 GM103533, R01 MH067880, UCLA/NHLBI Proteomics Centers (HHSN268201000035C). Funding for BBO has been provided by NIH AR049446.

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FIGURE LEGENDS

FIGURE 1. Pax7 stability is regulated by the proteasome during myogenesis.

A, Proteasome inhibition results in myogenin (Myog) and Pax7 co-expression (arrows) in adult mouse primary myoblasts (pMbs). Syndecan-4 (Sdc-4) expression was used as lineage marker. Scale bar: 10 μm. Right panel: quantification (three separate experiments; (mean ± s.e.m.)) of percentage of Myog(+)/Pax7(+) cells from the total Sdc-4(+) population; Student's t test *p<0.005.

B, Western Blot analysis of Pax7 and myogenin expression in 48 h cultured pMbs treated as in A.

C, Pax7 levels are significantly increased upon proteasome inhibition during differentiation commitment in C2C12 myoblasts. Myogenin (Myog): differentiation marker, tubulin (Tub): loading control. Right panel: quantification of Pax7 fold change (mean ± s.e.m.) of three separate experiments. One way ANOVA *p<0.005.

D, Epoxomicin mediated proteasome inhibition recapitulates Pax7 accumulation in differentiating C2C12 myoblasts. MG132 effect is shown as positive control. Right panel: quantification of Pax7 fold change (mean ± s.e.m.) of three separate experiments for 1μM epoxomicin and 25 μM MG132 treatments. One way ANOVA *p<0.005.

E, Proteasome inhibition results in Pax7 nuclear accumulation. Pax7 expression was determined by Western blot in cytoplasmic and nuclear fractions: (tubulin: cytoplasmic marker; histone deacetylase 2 (HDAC2): nuclear marker), Right panel: quantification of Pax7 protein levels normalized to HDAC2 (mean ± s.e.m.) of three separate experiments. Student's t test *p<0.005. MG132 was used at 25μM in **B**,**C** and **E**.

FIGURE 2. Pax7 is ubiquitinated during myoblast differentiation.

A, Bimolecular fluorescence complementation (BiFC) shows Pax7 ubiquitination in living cells. C2C12 myoblasts were transfected with the indicated constructs and treated with MG132 10⌈M, 6 h prior to live-cell imaging. Arrows indicate positive BiFC. Right panel: quantification (three separate experiments) of BiFC positive cells from total mRFP positive population. For Pax7-VC/Ub-VN complementation, this analysis shows 16,8 ± 8,4 % BiFC

positive cells in the DMSO condition and 60,3 ± 5,2 % in the MG132 condition. Positive control bFos-VC–bJun-VN complementation shows 92,3 ± 3,8% and 98,1± 1,9% for DMSO and MG132 treatment respectively. One way ANOVA *p<0.005.

B, Pax7 is ubiquitinated during myoblast differentiation. C2C12 cells were transfected with Pax7 and myc-6xHis-ubiquitin, induced to differentiate for 48 h and treated with MG132 10 μM 6 h prior to cell lysis. Denaturing Ni-NTA pulldown, followed by Western blot shows ubiquitinated Pax7 (bracket). Two Western blot examples for HA-Ub and Pax7 antigens are shown. Panels are aligned regarding molecular weights to show size differences between Pax7 (arrows) and Ub-Pax7 (arrowheads) after primary antibody stripping.

C, Pax7 is ubiquitinated *in vitro* (iUb). Purified GST-Pax7 or GST-only proteins were incubated with S-100 HeLa cell-extracts plus or minus energy-supply (ERS), followed by GST pull-down and Western blot for HA-tag. Arrowhead indicates ubiquitinated Pax7 species and arrow indicated GST-Pax7.

D, Pax7 can be monoubiquitinated during myogenic conversion. C3H10T1/2 cells were co-transfected with MyoD, Pax7 plus WT or mutant HA-Ub. Denaturing-IP for Pax7 followed by Western blot for HA show equivalent monoubiquitinated Pax7 species in all conditions (arrowhead).

E, Pax7 C-terminus is important for Pax7 proteasomal regulation. Upper panel: Experimental strategy. Lower panel: FL, ΔN and ΔHD Pax7 but not ΔC Pax7 levels are increased upon proteasome inhibition in differentiating (24 h) MyoD-converted C3H10T1/2 cells. GFP: transfection/loading control.

FIGURE 3. Nedd4 interacts with and regulates Pax7 stability in muscle precursors.

A, Upper panels: Indirect IF for laminin (LN) or Pax7 staining and Nedd4 in adult mouse TA cross-sections, show that Nedd4 is expressed in cells located in a SC position (arrows). Asterisk indicates that interstitial cells may also express Nedd4. Scale bar: 100 μm. Lower panels: Nedd4 is expressed in myofiber-associated SCs (arrows) fixed immediately upon isolation (0.5 h) or after 72 h in culture. 100% of 0.5 h (n=17) and 100% of 72 h (n=123) cultured SCs are Nedd4 positive. Scale bar: 10μm.

B, Nedd4 is expressed during differentiation of C2C12 myoblasts. Indirect immunofluorescence and confocal microscopy show that Nedd4 is expressed at similar levels in proliferating or differentiating C2C12 myoblasts. Scale bar: 10 μm.

C-D, Nedd4 physically interacts with Pax7 during C2C12 myoblast differentiation, as detected via *in vitro* GST pull-down utilizing purified GST-Pax7 and Nedd4 (C, arrowhead) or co-immunoprecipitation (D, arrowhead). Note that in D, Pax7-Nedd4 interaction occurred preferentially in differentiating cells (24-48 h).

E, Nedd4 negatively regulates Pax7 levels. MyoD-converted C3H10T1/2 were co-transfected with Pax7 plus Nedd4 siRNA (or control) and induced to differentiate for 48 h. Nedd4 knockdown results in a significant increase of Pax7 levels in transfected cells. Lower panel: quantification of Pax7 fold change (mean ± s.e.m.) after Nedd4 siRNA treatment. Student's t test *$p<0.005$.

F, Nedd4 ubiquitinates Pax7 *in vitro*. Purified Pax7 was incubated with E1, UbcH5B, HA-ubiquitin and energy supply with or without immobilized GST-Nedd4 protein. Ubiquitinated Pax7 was generated only in presence of Nedd4 (arrowheads) and energy supply. Controls for Nedd4, Pax7 and GST present in the supernatants are shown.

FIGURE 4. **Nedd4 localization is critical for the control of Pax7 stability**.

A, Nedd4 is preferentially located to cytoplasm in C2C12 cells. Proliferating and differentiating C2C12 cells were subjected to subcellular fractioning and Nedd4 levels were analyzed by Western blotting.. Lower panel: quantification of Nedd4 protein normalized to total cytoplasmic or nuclear protein, respectively (mean ± s.e.m.). One way ANOVA *$p<0.005$.

B, Nedd4 interacts with Pax7 at the cell nucleus; indicated by IP analysis from isolated subcellular fractions.

C, Inhibition of CRM-1/exporting-1 (leptomycin B, LMB) in C2C12 myoblasts, results in nuclear Nedd4 accumulation when compared with vehicle treated cells (EtOH); analyzed by confocal microscopy (Left panel) and Western blotting (Right panel mean ± s.e.m. of Pax7 fold change. Student's t test *$p<0.005$).

D, LMB treatment results in a reduction of Pax7 levels in C2C12 myoblasts. Right panel: Fold reduction of Pax7 levels in response to LMB treatment in proliferating and differentiating C2C12 cells (mean ± s.e.m.).

E, LMB induced Pax7 decline is dependent of proteasome activity. 48 h differentiating C2C12 cells were treated as indicated and analyzed via Western blotting (Right panel mean ± s.e.m. of Pax7 protein levels. One way ANOVA *p<0.005).

FIGURE 5. Nedd4 nuclear accumulation results in precocious muscle differentiation of C2C12 myoblasts.

A, LMB wash promotes Nedd4 cytoplasmic relocalization (arrowheads). C2C12 cells were treated for 6 h with LMB or vehicle (EtOH) prior to fixation or washed and cultured for additional 48 h prior to indirect immunofluorescence analysis. Scale bar: 10 μm.

B, Transient LMB treatment results in precocious myoblast differentiation. Cells were treated with LMB or vehicle (EtOH) for 6 h and either fixed immediately or washed and maintained in culture for 48 h (growth or differentiation conditions, as schematized) prior to fixation. LMB treatment resulted in a significant increase of myogenin positive cells in both conditions. Lower panel: quantification of myogenin positive cells per field (mean ± s.e.m.). One way ANOVA *p<0.005.

C, LMB mediated Pax7 protein reduction is dependent on Nedd4. Left panel: C2C12 cells were transfected with control (Ctrl) or Nedd4 siRNA and treated with vehicle or LMB 6 h prior to cell fixation. As expected, LMB treatment resulted in a Nedd4 nuclear accumulation concomitant to a decrease Pax7 protein levels (arrows). This phenotype is reversed by Nedd4 knock down (arrowheads). Right panel: Fold of reduction in Pax7 protein levels (mean ± s.e.m.) is significantly reduced by siNedd4 transfection. Student's t test *p<0.005.

EXPANDED VIEW FIGURE LEGENDS

FIGURE E1. Pax7 interacts with UPS-related proteins during myogenesis.

A, (i), General strategy for identifying Pax7-interacting proteins by mass spectrometry. (ii) Approximate equimolar Pax7 and MyoD expression levels were confirmed via Western blotting (Lower panel, highlighted box). (iii) MG132 treatment results in an increase of ectopic Pax7 protein levels. C3H10T1/2 cells were transfected with Pax7 and MyoD at the indicated ratio and induced to differentiate for 48 h prior to vehicle or MG132 treatment and Western blotting. GFP was used as a transfection control. (iv) C2C12 cells were transfected with Pax7-VC and cells were induced to differentiate for 48 h prior to vehicle or MG132 treatment and Western blotting. GAPDH protein was used as a loading control.
B, (i) Identified Pax7 interacting candidates are detailed. Note that, as described previously {Olguin:2007fs}, MyoD-Pax7 interaction was also detected in this analysis (*). Black box: Pax7-interacting candidates involved in protein turnover regulation via UPS. (ii) *In vitro* pull-down assays using purified recombinant GST-Pax7 and [^{35}S]-methionine labeled *in vitro*-translated candidates were performed to validate mass spectrometry data. A subset of these candidates (bold) was enriched in the GST-Pax7 pull-down fraction, suggesting direct physical interaction.

FIGURE E2: Bimolecular fluorescence complementation (BiFC) shows Pax7 ubiquitination in C2C12 cells.

A, Upper panel: Schematic representation of the principle used in this assay. bFos-bJun or Pax7-Ubiquitin interaction partners are fused to complementary non-fluorescent fragments of Venus protein (VC and VN). Venus fluorescence reconstitution is mediated by partner protein's interaction. Lower panel: BiFC in living C2C12 cells. For quantifications, cells transfected with bFos-VC/bJun-VN or Pax7-VC-Ubiquitin-VN were classified into two categories, BiFC positive (arrows) or BiFC negative cells. Scale bar: 10µm.
B, Pax7 ubiquitination is observed in cell nucleus via BiFC in fixed C2C12 cells. C2C12 cells were transfected with bFos-VC/bJun-VN or Pax7-VC/Ubiquitin-VN and treated with MG132

6 h prior to cell fixation, mounting and fluorescence microscopy analysis. Arrows indicate positive BiFC cells. Scale bar: 10µm.

FIGURE E3

A. Nuclear export inhibition induces 14-3-3 proteins nuclear accumulation. Proliferating C2C12 cells were treated with vehicle or 50 nM Leptomycin B (LMB) 6 h prior to cell fixation and immunofluorescence analysis for total 14-3-3 isoforms (rabbit polyclonal anti pan 14-3-3 antibody (K-19) Santa Cruz Biotechnology, Santa Cruz, CA, USA at 1:100). Note that LMB treatment results in strong nuclear accumulation of 14-3-3 protein (arrows) Scale bar: 10µm.

B, GST-Nedd4 and GST-only are retained by glutathione agarose beads. Eluates obtained from beads containing GST-Nedd4 or GST-only proteins were separated by centrifugation from Pax7 *in vitro* ubiquitination reaction and analyzed by Western blot. Note that ubiquitinated high molecular weight proteins (probably Nedd4 self-ubiquitinated species) are observed only in the presence of GST-Nedd4.

FIGURE E4: Leptomycin B (LMB) pretreated C2C12 cells show precocious Myosin heavy chain (MyHC) expression.

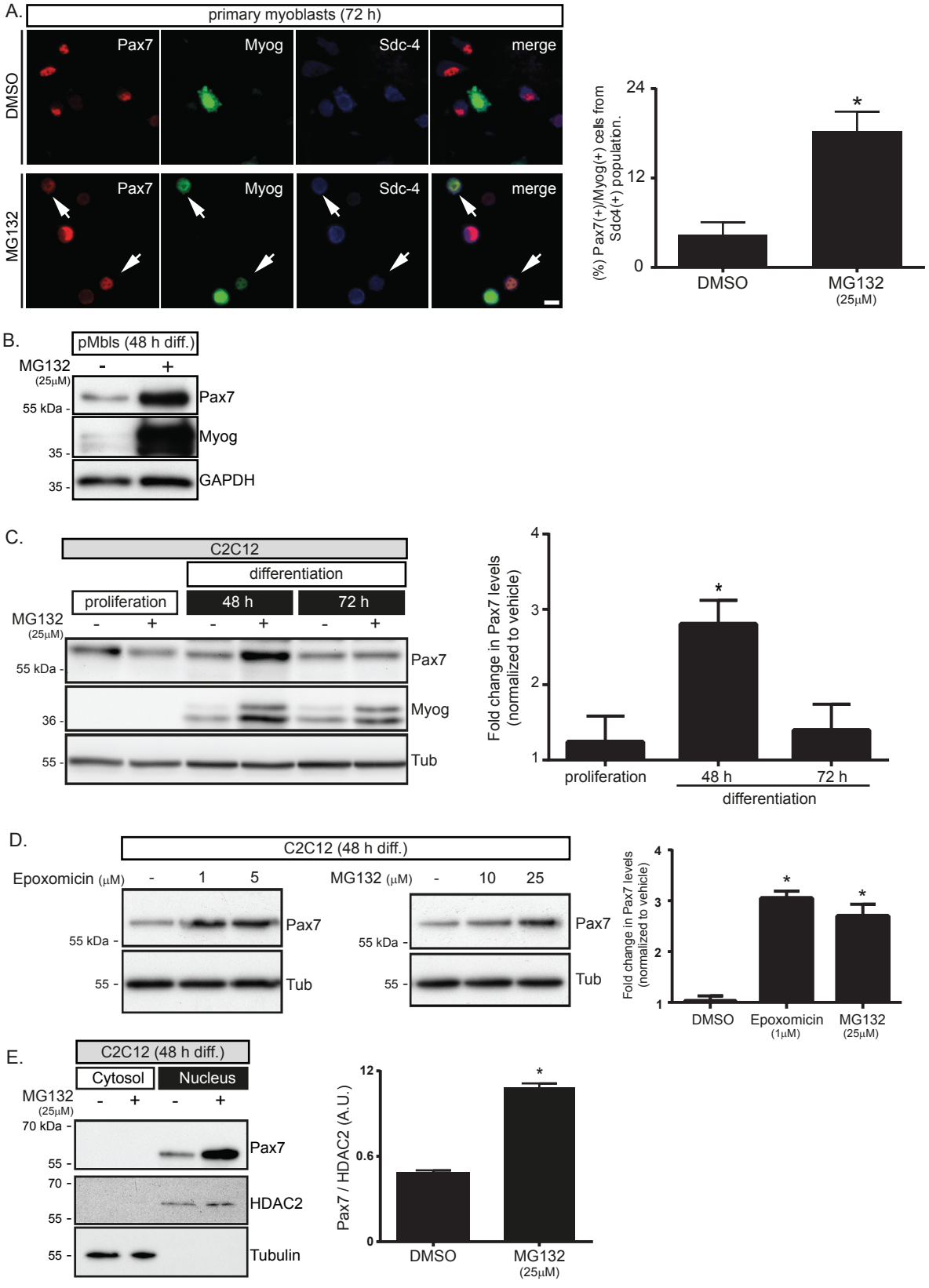
C2C12 cells were treated with vehicle or 50 nM LMB 6 h, washed and induced to differentiation for 48 h prior to fixation and immunofluorescence analysis to determine MyHC (monoclonal anti-MyHC antibody (MF-20) DSHB, Iowa City, IA, USA at 1:5) and Myogenin expression. Note that LMB treated cells show an increased number of MyHC positive cells compared to control (arrows). Note that it is possible to observe a higher number of Myogenin(+)/MyHC(-) cells in the control condition (arrowheads). Despite cells expressing MyHC are determined to terminal differentiation, no fusion is observed due to the short experimental time windows. Scale bar: 50µm. Lower panel: Quantification of the MyHC positive cells per field. (mean ± s.e.m.) after LMB treatment. Student's t test *$p<0.005$.

EXPANDED VIEW MATERIALS AND METHODS

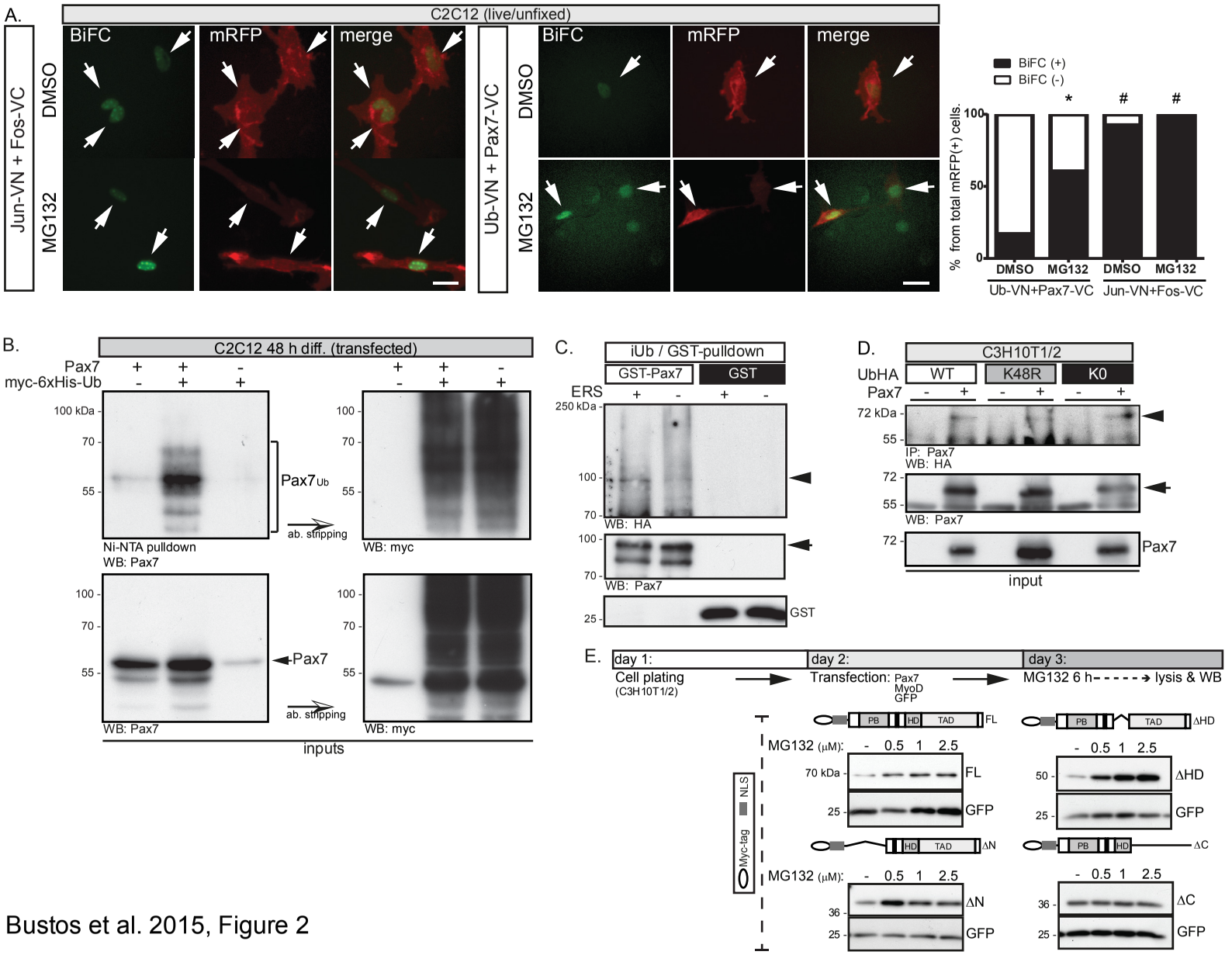
GST fusion proteins

pGEX-Pax7 and pGEX-mNedd4 plasmids were generated by directional subcloning of Pax7FL from pCDNA3-mycNLS-Pax7 or mNedd4 from pCDNA3-mNedd4 construct (kind gift from Dr. Sharad Kumar, Adelaide, Australia) to pGEX-4T1 plasmid (GE, Little Chalfont, BUX, UK) using BamHI and XhoI restriction sites. These plasmids were used to transform BL21(DE3) *E. coli*. Bacterial inocula were diluted 1:100 into 200 ml of Luria Bertani (LB) supplemented with 100 µg/ml ampicillin and grown at 37ºC up to A_{600}= 0.4. Cultures were then incubated over night at 15ºC in presence of 0.2 mM IPTG. Cells were lysed in TKET buffer (10 mM Tris-HCl Ph 7.5, 100 mM KCl, 0.1 mM EDTA, 0.05% Triton X-100 and protease inhibitors) at 4ºC. After sonication, 0.5% Triton X-100 was added and cell debris was removed by centrifugation at 11000 rpm. GST proteins were then captured by incubation of the supernatant with 250 µl per reaction of glutathione sepharose beads (Thermo Fisher Scientific, Rockford, IL, USA) for 2 h at 4ºC. Beads were recovered by centrifugation at 1000 rpm and washed 6 times in TKET buffer prior to elution. GST-Pax7 or GST-Nedd4 fusion proteins were eluted in 1ml of elution buffer (100 mM Tris-HCl pH 8.8, 100mM KCl, 20 mM glutathione) and then dialyzed over night against TKET buffer supplemented with 10% glycerol. Untagged Nedd4 or Pax7 were obtained using the thrombin cleavage capture kit (EMD Millipore, Billerica, MA, USA) according to manufacturer instructions. Supernatant protein concentration was determined by micro BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA) and the presence of the purified protein was tested by SDS-PAGE

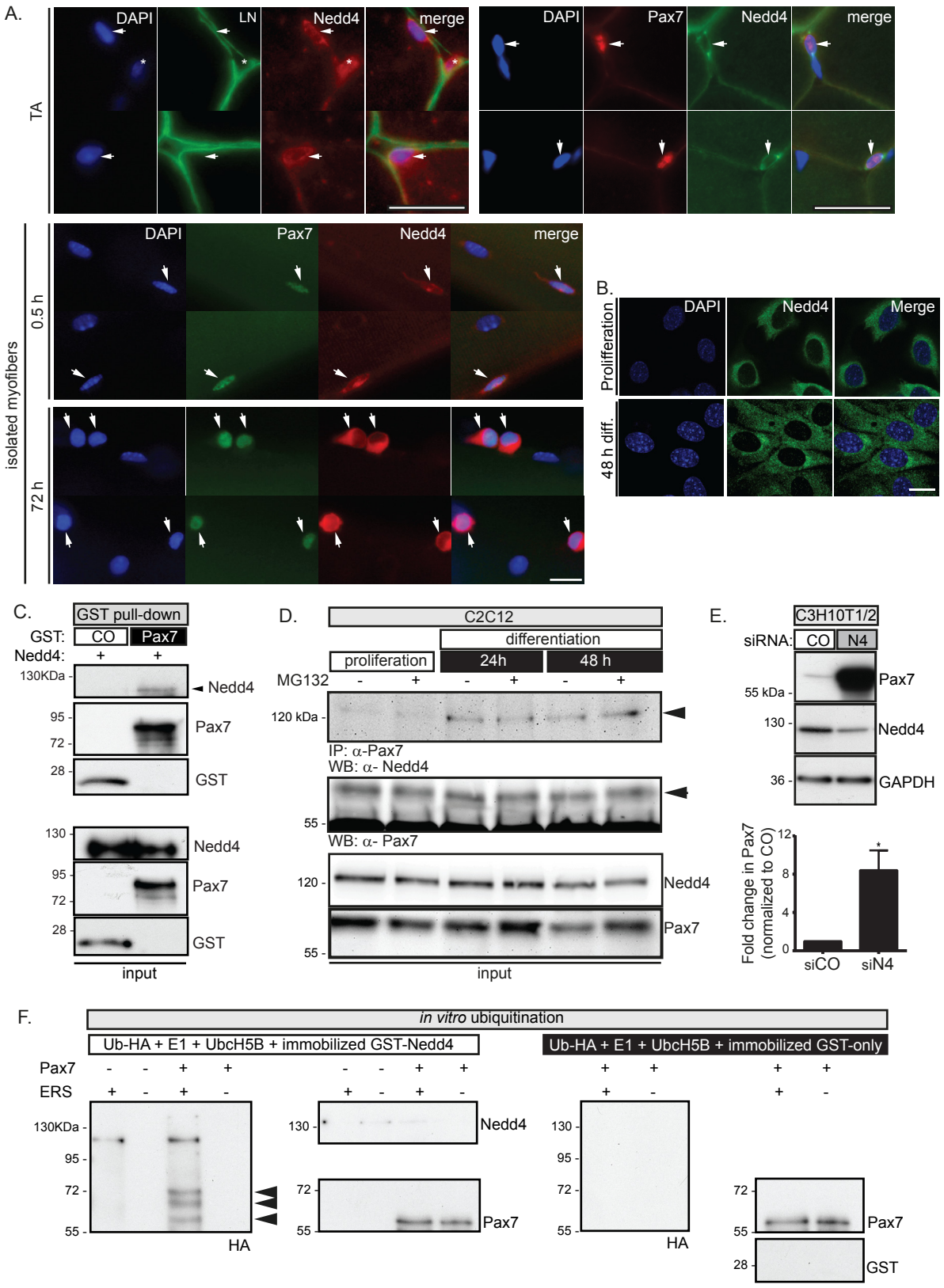
electrophoresis and gel code blue stain assay (Thermo Fisher Scientific, Rockford, IL, USA) or Western blot. All proteins were aliquoted and stored at -80ºC until use.



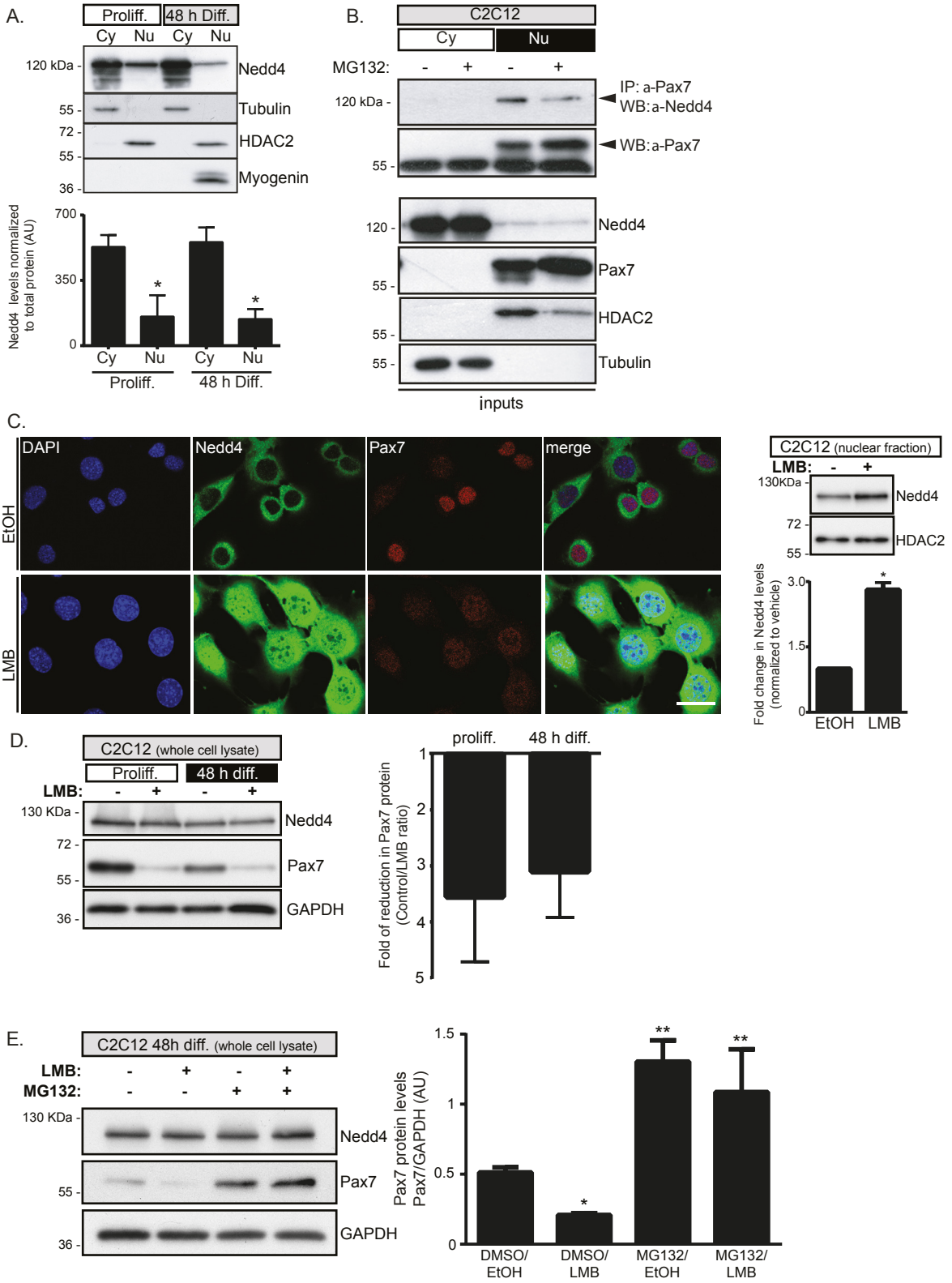
Bustos et al. 2015, Figure 1



Bustos et al. 2015, Figure 2



Bustos et al. 2015, Figure 3



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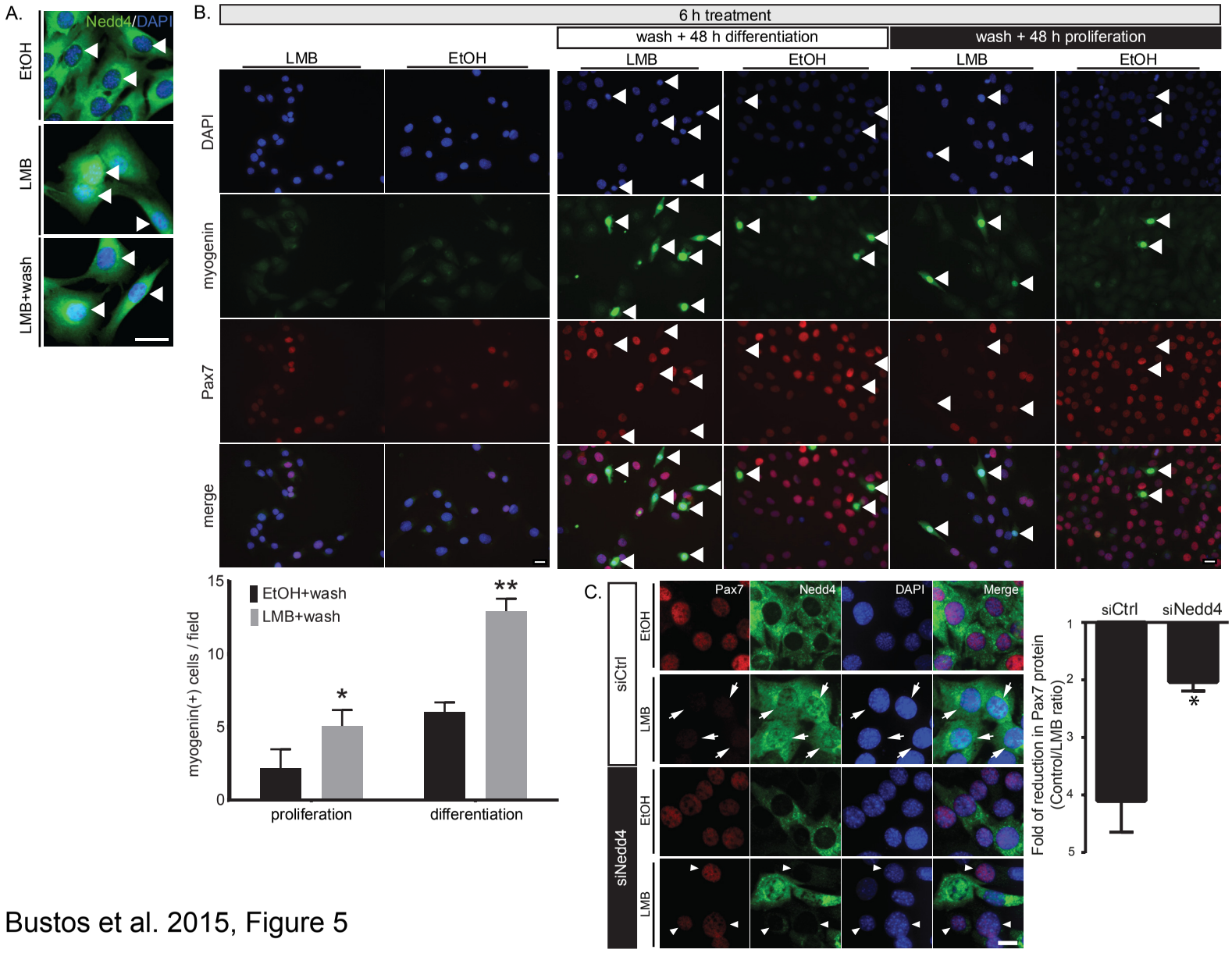
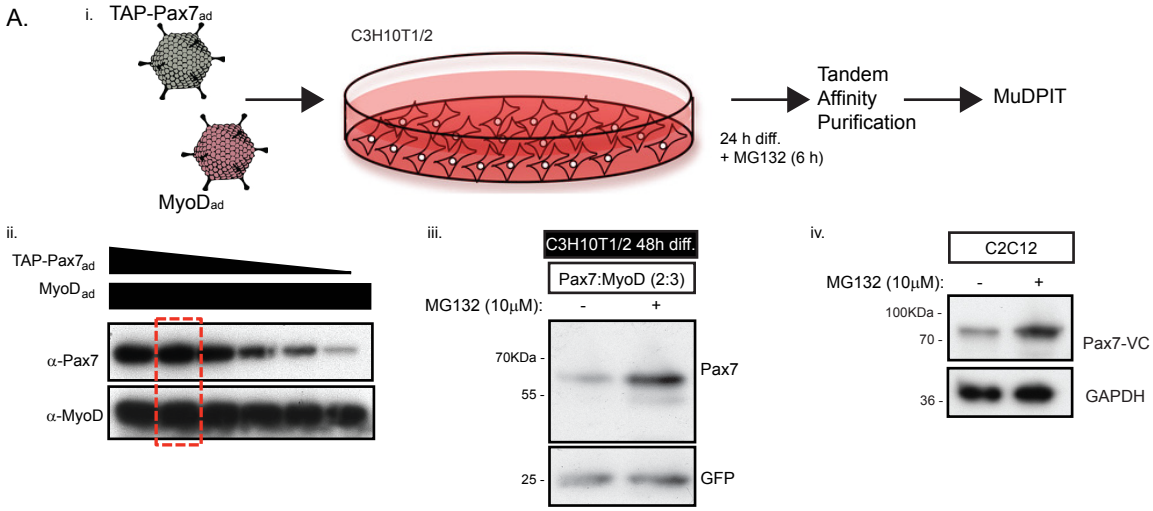


Bustos et al. 2015, Figure 5

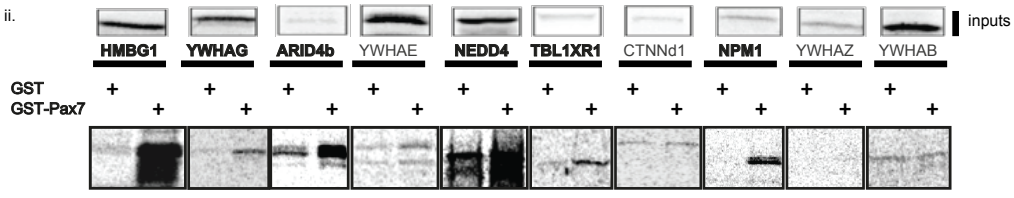
A.



B. i.

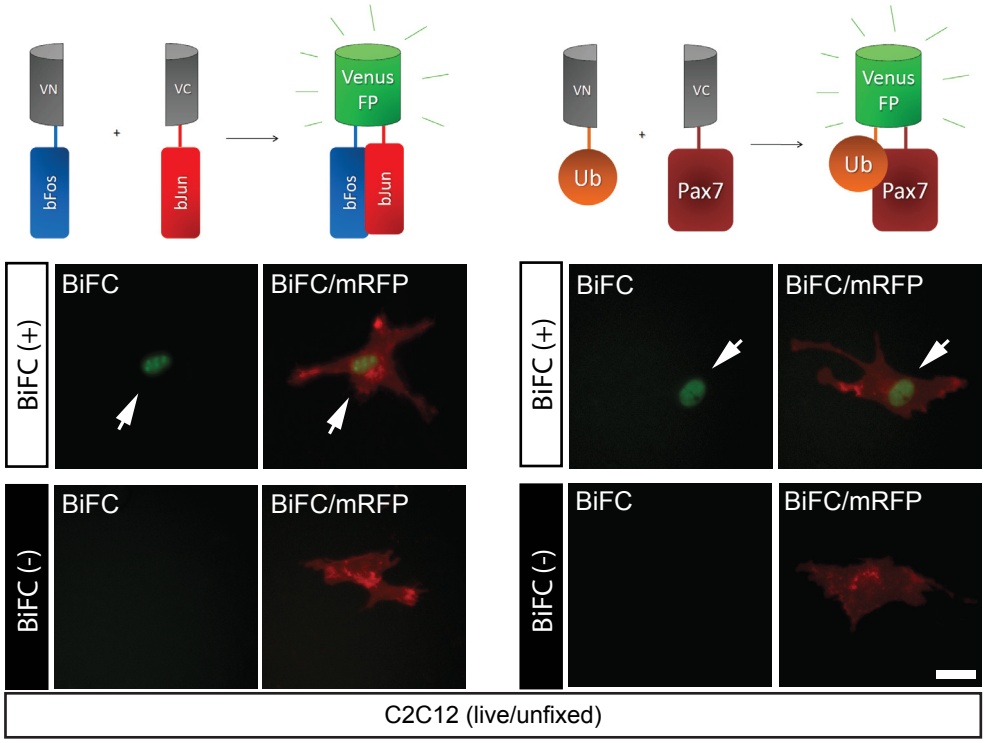
Selected Interacting Pax7 proteins identified by MuDPIT

Abbreviature	Protein name	Gene ID
CTNNd1	Catenin delta	12388
NPM1	Nucleophosmin	18148
NCOR2/SMRT	Nuclear receptor corepressor 2	20602
TBL1XR1	F-box-like/WD-repeat protein	81004
HMBG1	high mobility group box 1	15289
ARID4B	AT rich interactive domain 4B (RBP1-like)	94246
YWHAZ	14-3-3 protein zeta/delta	22631
YWHAB	14-3-3 protein beta/alpha	54401
YWHAG	14-3-3 protein gamma	22628
YWHAE	14-3-3 protein epsilon	22627
MyoD*	Myoblast determination factor1	17927
Uba1	Ubiquitin-like modifier activating enzyme 1	22201
Usp7	Ubiquitin specific peptidase 7	252870
Psma3	Proteasome (prosome, macropain) subunit, alpha type 3	19167
Nedd4	Nedd4 neural precursor cell expressed, developmentally down-regulated 4	17999

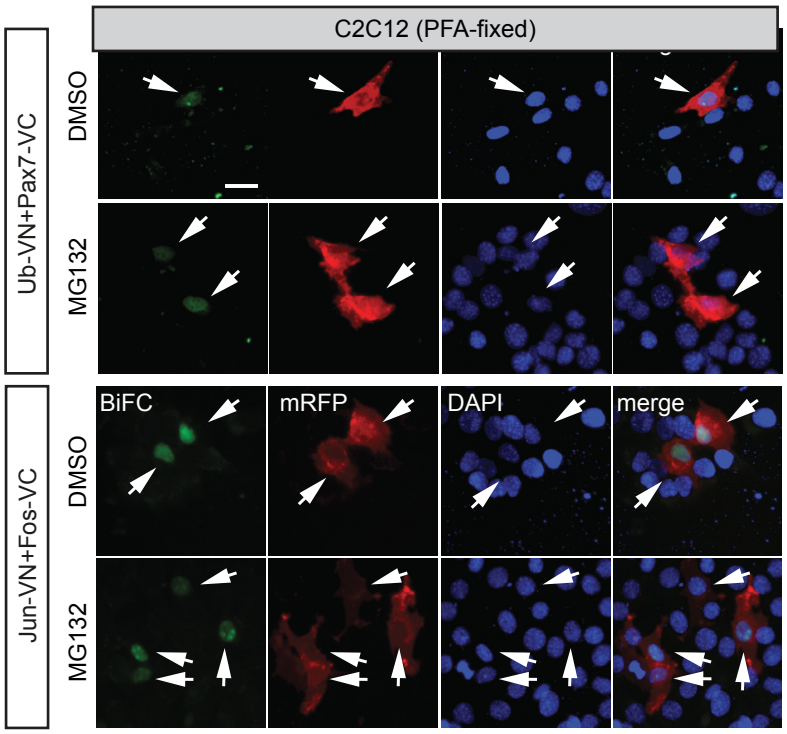


Bustos et al. 2015, Figure E1

A.



B.



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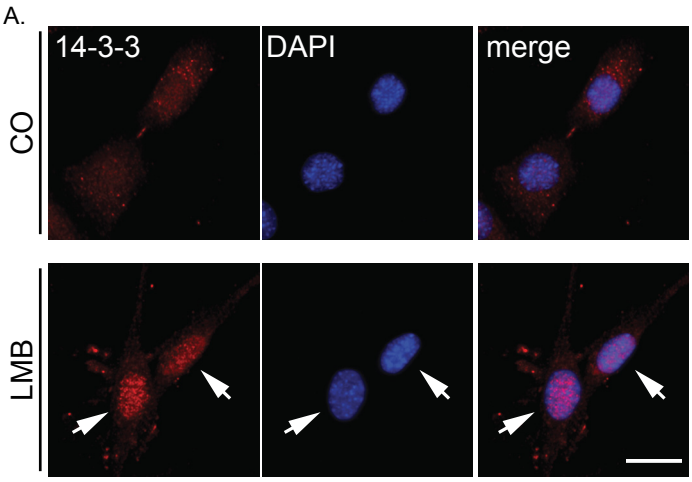

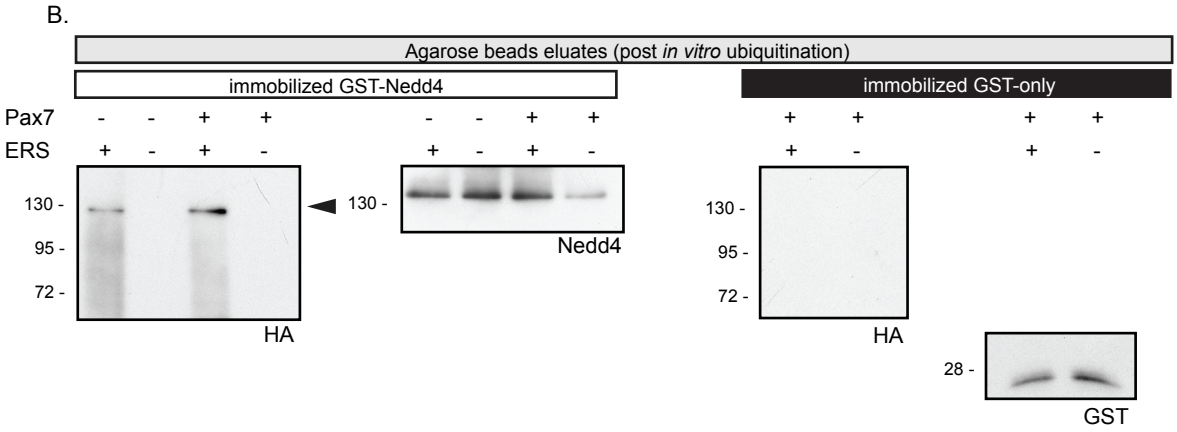

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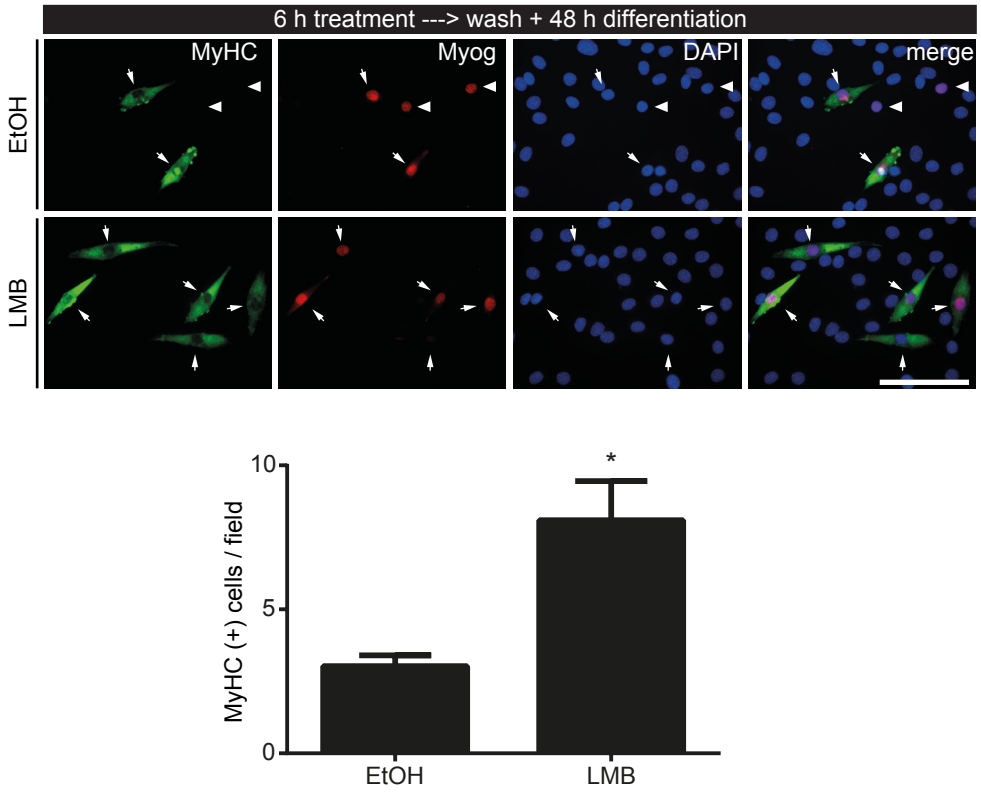

Bustos et al. 2015, Figure E4.

DISCUSIÓN GENERAL.

Pax7 es degradado por el UPS nuclear en precursores musculares. Un nuevo mecanismo de regulación de Pax7 en los mioblastos en diferenciación.

La presente tesis de doctorado introduce al sistema ubiquitina-proteasoma (UPS) como un nuevo regulador de la expresión de Pax7. Utilizando varios modelos celulares hemos demostrado que este factor de transcripción es sujeto a ubiquitinación mediada por la E3 ligasa Nedd4 (Fig. 3) y degradación proteasomal durante etapas tempranas de la diferenciación de células precursoras musculares (Fig. 1, Fig A1).

Aquí describimos por primera vez que Pax7 es regulado por el UPS en mioblastos en diferenciación en un proceso que puede influenciar las decisiones sobre el destino de estas células. Pax7 reprime la miogénesis inhibiendo la función de MyoD en un circuito regulatorio en que MyoD reprime recíprocamente los niveles de expresión y por tanto la función de Pax7 (Joung et al., 2014; Kumar et al., 2009; Olguin et al., 2007). Se ha sugerido que pequeños desbalances en la razón de los factores Pax7 y MyoD pueden ser críticos para las decisiones sobre el destino de estas células hacia la diferenciación o autorrenovación (Olguín and Pisconti, 2012). En este escenario, la regulación postranscripcional y principalmente la regulación postraduccional de Pax7 pueden jugar un rol clave para definir el destino de las células satélite durante la regeneración muscular.

Bajo este modelo, la ubiquitinación y degradación de Pax7 mediada por el proteasoma disminuiría la razón Pax7:MyoD, permitiendo que se induzcan eventos moleculares que terminan en la diferenciación muscular. De esta manera, al disminuir la proteína Pax7, se inhibiría la regulación negativa que Pax7 ejerce sobre MyoD y así se reduciría la

ubiquitinación y degradación de MyoD. También disminuiría la inducción del blanco transcripcional de Pax7 Id3, que inhibe la actividad de MyoD. Concomitantemente, aumentarían los niveles de MyoD y por tanto la inducción de sus blancos transcripcionales como miogenina, la cual produce una disminución en los niveles de Pax7. Esta retroalimentación positiva terminaría en la diferenciación terminal de los precursores musculares.

A nivel postranscripcional, uno de los posibles mecanismos que resultan en la disminución de Pax7 durante la diferenciación muscular, es la participación de micro ARNs (Ge and Chen, 2011). Se ha descrito que los micro ARN miR-1, miR-206 y miR-486 inhiben la traducción de Pax7 en el contexto miogénico promoviendo la diferenciación muscular (Chen et al., 2010; Dey et al., 2011). Particularmente la expresión de miR-206 y miR-486 es inducida por MyoD en células que se comprometen al linaje muscular (Dey et al., 2011).

Los mecanismos postraduccionales podrían permitir cambios rápidos en la razón de proteína Pax7/MyoD, lo que dependiendo del destino final de la célula, se reforzarían y mantendrían por mecanismos transcripcionales y/o post transcripcionales llevando a la célula hacia la diferenciación o autorrenovación (Olguín and Pisconti, 2012). Es por esto que nosotros nos hemos interesado en estudiar la regulación postraduccional de la proteína Pax7. Esto además se sustenta en la observación de que comparativamente, la caída de la proteína Pax7 comienza antes de que la disminución de los niveles de mensajero en células C2C12 (Dey et al., 2011). La evidencia indica también que interacciones del tipo proteína-proteína definen la función de Pax7 durante la miogénesis (Diao et al., 2012; McKinnell et al., 2008; Olguin et al., 2007).

Como ocurre para otros factores de transcripción (Boutet et al., 2007; Boutet et al., 2010; Gu and Zhu, 2012) Pax7 presenta diversas modificaciones postraduccionales. Por ejemplo, en nuestro laboratorio se ha demostrado recientemente que la actividad de caspasa-3 puede controlar los niveles de Pax7 en mioblastos en diferenciación, en un mecanismo que podría funcionar en paralelo o en cooperación al aquí descrito (Olguín, 2011). Además, nos encontramos actualmente explorando la función de fosforilaciones mediadas por la Caseín quinasa 2 en la regulación de la función de Pax7 durante la miogénesis (Natalia González, memoria de investigación de la carrera de bioquímica, Pontificia Universidad Católica de Chile, 2014). Por otro lado, otros grupos han demostrado que Pax7 es SUMOilado en mioblastos. La SUMOilación modula la capacidad de Pax7 de inhibir la miogénesis (Luan et al., 2012). En este escenario y en vista de los resultados obtenidos en esta tesis acerca de la ubiquitinación y degradación proteasomal de Pax7 (Fig. 1-3), es posible que la expresión de Pax7 sea altamente regulada durante la miogénesis por una combinación de diferentes modificaciones postraduccionales que podrían definir las actividades de Pax7 sobre sus blancos transcripcionales o proteínas interactuantes. En este sentido, la identificación de los residuos de lisina implicados en la ubiqutinación de Pax7 será muy importante para evaluar la relevancia funcional de esta modificación y su relación con los dominios funcionales de esta proteína. Esto también nos permitiría estudiar cómo esta modificación podría resultar en cambios conformacionales en estructura tridimensional de Pax7 y como podría relacionarse en una red compleja con otras modificaciones postraduccionales influyendo en la función de Pax7 en las células satélite (Fig. D2A).

La E3 ligasa Nedd4 interactúa con Pax7 y cataliza su ubiquitinación. ¿Un mecanismo para la regulación del destino de las células satélite durante la regeneración muscular?

Aquí hemos demostrado por diversas estrategias que la E3 ubiquitina ligasa Nedd4 interactúa bioquímicamente con Pax7 y cataliza su ubiquitinación (Fig. 3, Fig A3). Nedd4 se expresa en las células satélite del músculo esquelético adulto y también en los modelos celulares analizados (Fig. 3). Nedd4 interactúa con Pax7 *in vivo* e *in vitro*, esta interacción es dependiente del dominio amino terminal de Pax7 (Fig. A3). Además, a través de un ensayo de ubiquitinación *in vitro*, demostramos que Nedd4 cataliza la ubiquitinación de Pax7 (Fig. 3F). Dado que la enzima E3 ligasa es la que define la especificidad de sustrato de la reacción de ubiquitinación, la identificación de Nedd4 como E3 específica de Pax7 supone un paso importante para entender la regulación de Pax7 y junto con ello plantear a esta enzima como un blanco para la manipulación de las decisiones del destino celular de los precursores musculares.

En la presente tesis, mediante silenciamiento mediado por ARN de interferencia y tratamientos farmacológicos con la droga LMB, hemos demostrado que Nedd4 es un regulador negativo de la proteína de Pax7 (Fig. 3E, 4C-E, 5C) y que la localización forzada de Nedd4 en el núcleo resulta en una diferenciación precoz mediada por la disminución transitoria de Pax7 (Fig. 5 y Fig. E4). Si bien estos resultados sugieren que Nedd4 actúa promoviendo la diferenciación muscular, se requieren estudios más detallados para establecer el requerimiento de la actividad de Nedd4 para la diferenciación y regulación del destino celular de los mioblastos. En este sentido, experimentos de silenciamiento o *knockout* de Nedd4 inducido específicamente en la población de células satélite podrán establecer

directamente (i) la función de Nedd4 en las células satélite quiescentes, (ii) funciones de Nedd4 en el citoplasma y sobre otros sustratos en estas células y (iii) el requerimiento de Nedd4 para la regeneración muscular (Figura D2B) (Felipe Cabezas, proyecto de tesis de doctorado, Programa de doctorado en Ciencias Biológicas mención Biología Celular y Molecular, Pontificia Universidad Católica de Chile, 2014).

Nedd4 cataliza la ubiquitinación de Pax7 ¿Existe regulación de la actividad de Nedd4 durante la diferenciación muscular?

Por lo general, la sobreexpresión de una E3 ligasa resulta en una disminución de su proteína sustrato (Zhu et al., 1999). Sin embargo, en nuestro modelo de estudio, la sobreexpresión de Nedd4 no afectó los niveles de Pax7 (Fig. A2). Es por esto que nosotros proponemos que la actividad de Nedd4 debía depender de algún tipo de regulación ya fuera de su actividad o localización.

El microambiente circundante (o nicho) de las células satélite cumple un papel regulatorio directo sobre la mantención y función de estas células (Bentzinger et al., 2013). Las células satélite establecen interacciones célula-matriz y célula-célula con diversos tipos celulares no musculares incluyendo fibroblastos, células endoteliales, progenitores fibro-adipogénicos, recibiendo y enviando señales que influyen en su comportamiento durante la regeneración muscular (Murphy et al., 2011). Así, los mecanismos que conectan la señalización proveniente del nicho de las células satélite con la actividad Nedd4 son desconocidos y podrían ser relevantes para entender la regulación Pax7 en diferentes etapas de la diferenciación muscular.

Esto parece posible en vista de reportes que indican que la actividad Nedd4 se puede regular a través de estímulos extrínsecos. Por ejemplo, Nedd4 es fosforilado en respuesta a la activación del receptor de FGF. Esta fosforilación activa Nedd4 antagonizando la autoinhibición mediada por su dominio C2 (Persaud et al., 2014). Este mecanismo podría influir en la función de Nedd4 que se describe la presente tesis de doctorado.

Mediante experimentos farmacológicos con la droga LMB, la que bloquea el exporte nuclear dependiente de exportina-1/CRM-1, pudimos determinar que la localización de Nedd4 en mioblastos es regulada. Tal como ocurre en células de otros linajes (Hamilton et al., 2001), Nedd4 es exportado activa y continuamente desde el núcleo al citoplasma en mioblastos (Fig. 4C, 5C). En consecuencia, el tratamiento con LMB resulta en la acumulación de Nedd4 en el núcleo, que es simultáneo a una disminución significativa en los niveles de Pax7 dependiente del proteasoma (Fig. 4C-E). Estos resultados nos indicaron que el exporte nuclear de Nedd4 es un mecanismo que regula la actividad de Nedd4 sobre Pax7, puesto que cuando se interrumpe la localización eddógena de Nedd4, los niveles de Pax7 disminuyen. Experimentos preliminares de nuestro laboratorio realizados en cultivos de miofibras *ex vivo* muestran que si se analizan células satélite recién extraídas LMB no tiene efecto. Esto indica que en esta etapa, Nedd4 se encuentra restringido al citoplasma. Por el contrario luego de 24 horas de cultivo, el tratamiento con LMB resulta en la acumulación nuclear de Nedd4 en las células satélite (Felipe Cabezas, proyecto de tesis de doctorado, Programa de doctorado en Ciencias Biológicas mención Biología Celular y Molecular, Pontificia Universidad Católica de Chile, 2014). Estos resultados indican que la entrada de Nedd4 al núcleo está regulada durante la activación de las células satélite. Esta regulación podría constituir un elemento importante para entender cómo se establece una regulación diferencial de Pax7 en mioblastos en

diferenciación y no en mioblastos que se encuentran en estado quiescente o etapas proliferativas.

Nedd4 induce la ubiquitinación y degradación de Pax7 en los mioblastos en diferenciación, ¿Es inhibido este mecanismo durante la autorrenovación de las células satélite?

La autorrenovación es un mecanismo fundamental para la mantención de la capacidad regenerativa de un tejido. Esta capacidad define a las células troncales (Smith, 2006) y está presente en las células satélite (Collins and Partridge, 2005; Moss and Leblond, 1971). Existe evidencia de que las células satélite pueden autorrenovarse mediante división asimétrica (Kuang et al., 2007) o divisiones simétricas (Le Grand et al., 2009). La evidencia sugiere que Pax7 es necesario para la autorrenovación de las células satélite y por lo tanto se requiere de altos niveles de esta proteína para mantener el estado indiferenciado de las células satélite (Günther et al., 2013; Rocheteau et al., 2012). El hecho de que Nedd4 se expresa en las células satélite quiescentes (Fig. 3A) sugiere que es necesario un mecanismo que inhiba su actividad sobre Pax7 en dicha condición.

Por ejemplo, está demostrado que en el caso de los progenitores neurales (NPCs), el factor de transcripción REST que promueve la autorrenovación, es regulado a nivel postraduccional por el UPS una vez que los NPCs inician su diferenciación terminal (Westbrook et al., 2008). Este mecanismo es antagonizado en las células que autorrenuevan por la deubiquitinasa HAUSP/Usp7, cuya expresión es inducida en células que se autorrenuevan manteniendo la población de NPCs indiferenciados (Huang et al., 2011). En dicho trabajo se plantea que este balance permite a las células troncales responder a estímulos

externos rápidamente desembocando cambios mediados por la regulación postraduccional de factores clave para la determinación del destino celular de las NPCs (Huang et al., 2011). Es posible que este corresponda a un mecanismo general que regula las decisiones de las células troncales. Si es así, en nuestro modelo de estudio, la actividad de Nedd4 podría ser regulada por la actividad de una enzima deubiquitinasa que promueve la autorrenovación de las células satélite en respuesta a estímulos externos determinados. La identificación de esta enzima y de dichos estímulos, podría entregar un mejor entendimiento de cómo el UPS regula el destino de las células satélite.

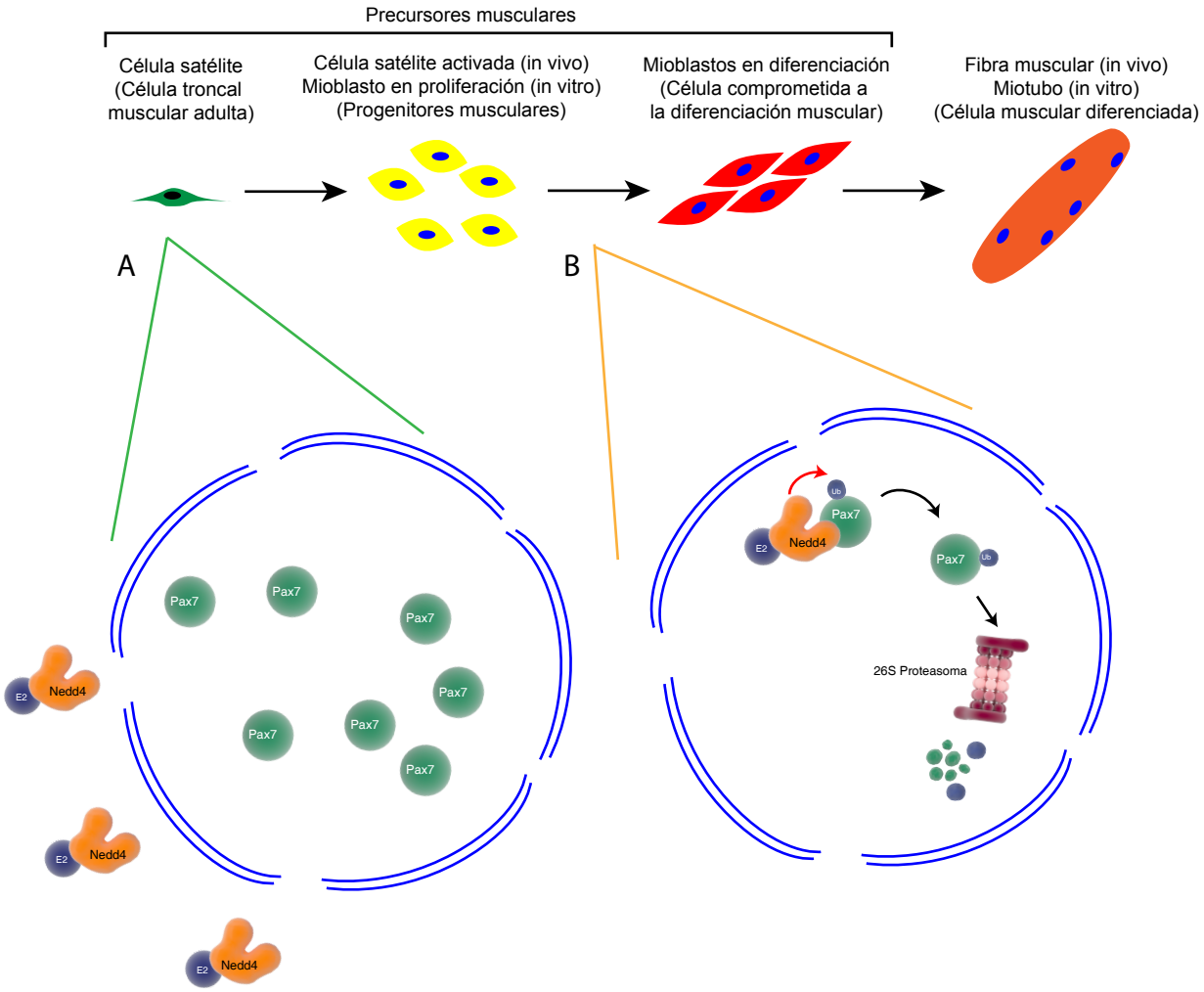

Figura D1: Modelo de trabajo.
Arriba: Esquema de la diferenciación de las células satélite durante el proceso de regeneración muscular (miogénesis adulta). Se indican los términos utilizados para nombrar a las células satélite y sus descendientes en las distintas etapas de la diferenciación *in vivo* o en modelos *in vitro*. Abajo, en base a los resultados de la presente tesis de doctorado se puede concluir que A. En células satélite quiescente Nedd4 se encuentra localizado fuera del núcleo y su función se encuentra inhibida permitiendo que Pax7 se encuentre en altos niveles ejerciendo su función. B. Nedd4 se localiza en el núcleo y cataliza la ubiquitinación de Pax7 induciendo su degradación en el proteasoma en etapas tempranas de la diferenciación de los precursores musculares adultos.

Proyecciones biomédicas de la función de Nedd4 en las células satélite musculares.

El conocimiento adquirido durante el desarrollo de esta tesis de doctorado podría tener importantes implicancias en nuestro entendimiento de la regulación de Pax7 por Nedd4 en las células satélite y los mecanismos que controlan las decisiones de estas células. Sin embargo, considerando los desafíos actuales en el estudio de las células satélite musculares, nuestros resultados entregan importantes proyecciones con relevancia en la medicina regenerativa:

Actualmente no existen aplicaciones biomédicas que tengan a Nedd4 como un blanco terapéutico. Nedd4 ha sido relacionado al desarrollo del cáncer funcionando como un proto-oncogen que promueve el crecimiento tumoral (Amodio et al., 2010; Hong et al., 2013; Jung et al., 2013; Wang et al., 2007; Yang et al., 2012; Zhang et al., 2013). Estos hallazgos han situado a Nedd4 como un posible blanco terapéutico interesante para combatir diversos cánceres (Ye et al., 2014). Desafortunadamente, el desarrollo de drogas moduladoras de las enzimas E3 del tipo HECT (Del inglés: *Homologous to E6AP carboxy terminus*) como Nedd4 se ha visto limitado por la falta de conocimiento acerca de su regulación y características estructurales que definen: i) su interacción con el sustrato y ii) la actividad de su dominio catalítico (Scheffner and Staub, 2007).

Aún así, los avances en esta dirección podrían no ser suficiente para resolver los efectos secundarios inherentes a la administración de drogas contra una enzima E3 a nivel sistémico, debidos a que estas enzimas pueden regular más de un sustrato en un determinado tipo celular y tejido (Scheffner and Staub, 2007). La información proporcionada por la presente tesis doctoral que sitúan a Pax7 como un sustrato de Nedd4 enel contexto miogénico no descarta la posibilidad de que Nedd4 posea otros blancos en estas células. La localización

predominantemente citoplasmática de Nedd4 (Fig. 4) nos lleva a pensar que esta enzima podría catalizar la ubiquitinación y regular la función de otros sustratos presentes fuera del núcleo. En consecuencia, se requiere conocimiento adicional acerca de otros efectos que podría tener Nedd4 sobre las células satélite y sobre la especificidad de Nedd4 sobre Pax7 en este contexto para evaluar potenciales aplicaciones biomédicas en torno a esta E3 ligasa.

Actualmente la terapia basada en el transplante de células satélite es una de las aproximaciones teóricamente más promisorias para el tratamiento de enfermedades degenerativas del músculo esquelético (Bentzinger et al., 2013). En este contexto, los esfuerzos de los investigadores están centrados en mejorar la expansión *in vitro* de las células satélite, su corrección si es necesaria y su incorporación al tejido dañado específicamente en el nicho de las células satélite, de tal manera de proveer una fuente sustentable de células troncales al tejido (Bareja and Billin, 2013). La identificación de factores que regulan el destino celular de los precursores musculares, como en este caso Nedd4, podría permitir mejoras en el desarrollo de la terapia basada en el transplante de estas células (Figura D2B) (Kuang and Rudnicki, 2008).

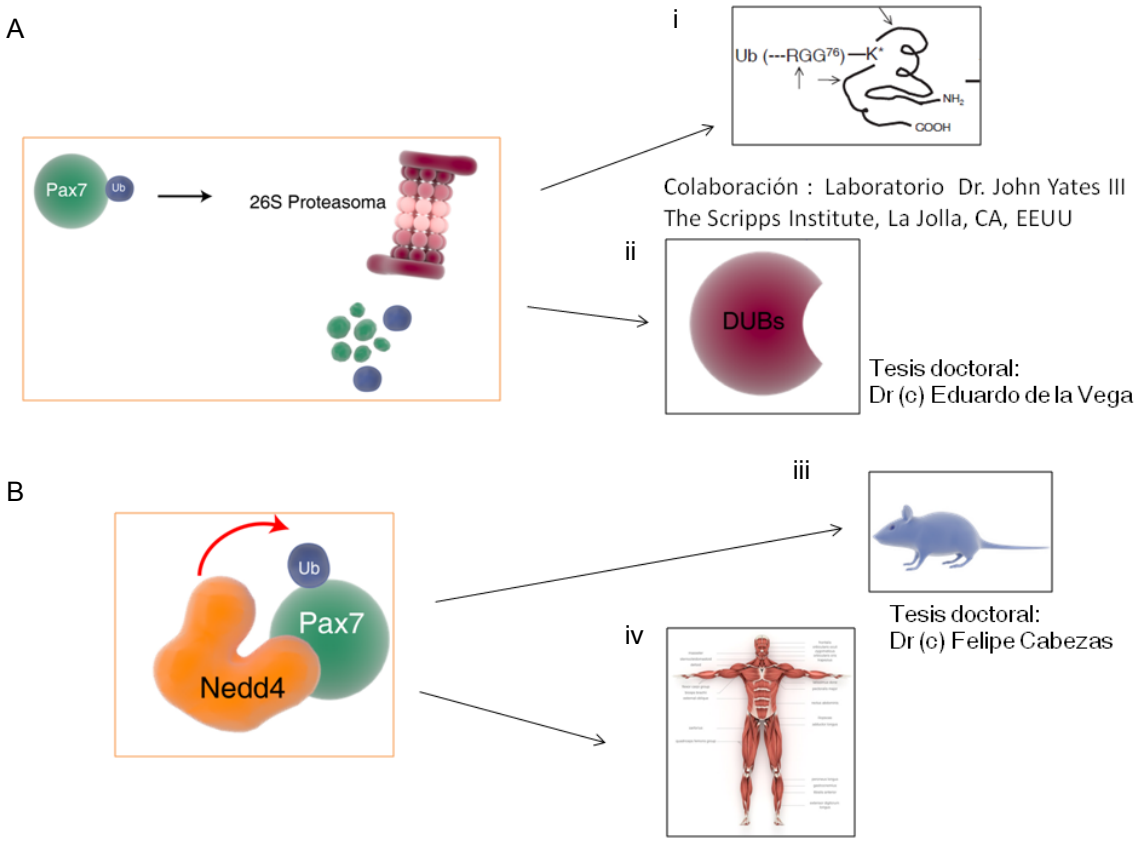

Figura D2: Proyecciones resultantes de la presente tesis doctoral:
A. Se esquematiza la conclusión de los resultados de esta tesis doctoral los que indican que el sistema ubiquitina proteasoma puede regular a Pax7 y el destino de las células satélite. En cuanto a esto se plantean dos proyecciones. (i) Identificar los residuos de lisina ubiquitinados en Pax7 de forma de poder estudiar la relevancia funcional de esta modificación en la función de Pax7. (ii) Estudiar la función de la deubiquitinasa Usp7 en la regulación de la diferenciación de los precursores musculares (Proyecto de tesis doctoral PUC, Eduardo de la Vega). B. Los hallazgos relacionados a la función de Nedd4 como un nuevo regulador de la expresión de Pax7 durante la diferenciación de las células satélite plantea proyecciones conducentes a (iii) Investigar la función de Nedd4 en la regeneración muscular en un modelo de ratón transgénico nulo inducible tejido específico en las células satélite (Proyecto de tesis doctoral PUC, Felipe Cabezas) y (iv) Nedd4 es un posible blanco farmacológico con posibles aplicaciones en el control del destino de las células satélite, necesario para las futuras terapias celulares basadas en células satélite para las enfermedades degenerativas del músculo esquelético.

CONCLUSIONES.

- Pax7 es ubiquitinado y degradado por el proteasoma nuclear en etapas tempranas de la diferenciación de precursores musculares adultos.
- La E3 ligasa Nedd4 se expresa en las células satélite musculares e interactúa con Pax7 durante la diferenciación de los precursores musculares adultos.
- Nedd4 cataliza la ubiquitinación de Pax7 e induce su disminución en precursores musculares adultos.
- El exporte nuclear de Nedd4 es un mecanismo que regula su actividad sobre Pax7.
- La función de Nedd4 como regulador de los niveles de Pax7 promueve la diferenciación muscular de los precursores musculares adultos.

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LEYENDAS DE FIGURAS ANEXAS

FIGURA ANEXA 1: La inhibición del proteasoma resulta en un aumento de Pax7 en mioblastos MM14 en diferenciación. Mioblastos MM14 fueron cultivados en condiciones de proliferación o de diferenciación por 24 o 96 horas y tratadas con MG132 25 μM 6 horas antes de su lisis y su posterior análisis mediante Western blot. GAPDH fue utilizado como control de carga. En este experimento se observa que los niveles de Pax7 aumentan en respuesta a la inhibición del proteasoma a en células a 24 horas de inducida la diferenciación. Este aumento no se observa en células en proliferación ni tampoco en mioblastos diferenciados por 96 horas.

FIGURA ANEXA 2: La sobreexpresión de Nedd4 no afecta la proteína de Pax7. A, células C3H10T1/2 fueron transfectadas con Pax7 y las cantidades indicadas de pCDNA3-mNedd4-flag. Mediante Western blot es posible observar que esta ganancia de función de Nedd4 no es suficiente para inducir una disminución de Pax7. **B**, mioblastos C2C12 fueron transfectados con plasmidio control o (pCDNA3) o pCDNA3-mNedd4-flag. La proteína fluorescente amarilla de membrana fue expresada mediante el vector EYFP-M y fue utilizada como marcador de Transfección. Mediante inmunofluorescencia es posible observar que la sobreexpresión de Nedd4 no afecta los niveles de Pax7 en las células transfectadas (cabeza de flecha). Se puede observar también que la localización de Nedd4-flag es predominantemente citoplasmática (flecha). Barra de escala: 10 μm.

FIGURA ANEXA 3: El dominio amino terminal de Pax7 es necesario y suficiente para su interacción con Nedd4. A, esquema de los mutantes de deleción de Pax7 fusionados a GST que fueron utilizados en este estudio. **B**, Los dominios amino y carboxilo terminal de Pax7 son necesarios para la interacción de Pax7 con Nedd4. Ensayo de interacción *in vitro* utilizando las proteínas de fusión purificadas indicadas, las cuales fueron incubadas por 20 horas en presencia de Nedd4 purificado, luego sometidos a un ensayo de GST-pulldown y analizados mediante Western blot. Se puede observar que Nedd4 interactúa con GST-Pax7 completo (FL) o GST-Pax7 que carece del homeodominio (ΔHD). **C**, el dominio amino terminal de Pax7 es suficiente para la interacción de este factor con Nedd4. Ensayo de interacción in vitro utilizando las proteínas de fusión indicadas las cuales fueron incubadas con Nedd4, sometidas a GST-pulldown y analizados mediante Western blot. Se puede observar que Nedd4 interactúa con GST-Pax7 completo (FL) y con el amino terminal de Pax7 (ΔOC), pero no interactúa con la proteína correspondiente al carboxilo terminal de Pax7 (ΔNHD).

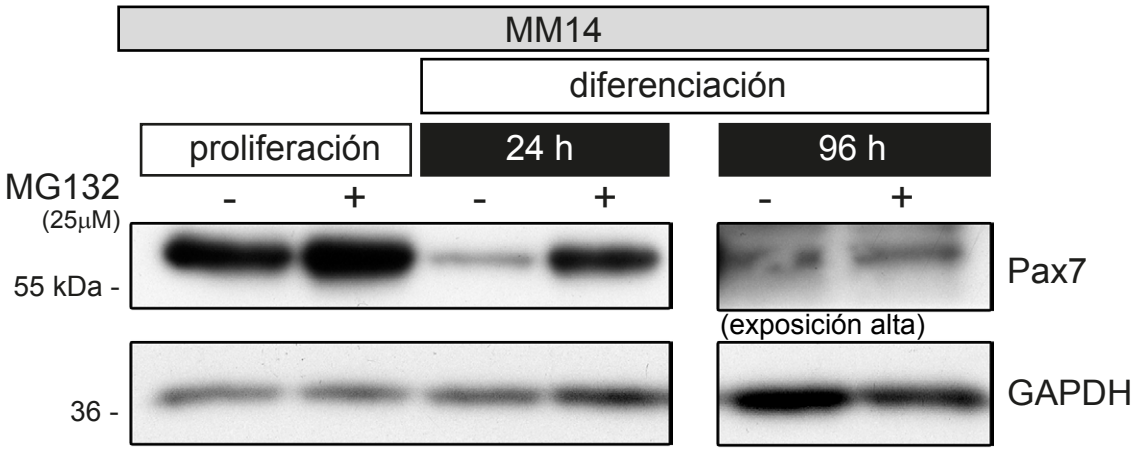


Figura anexa 1

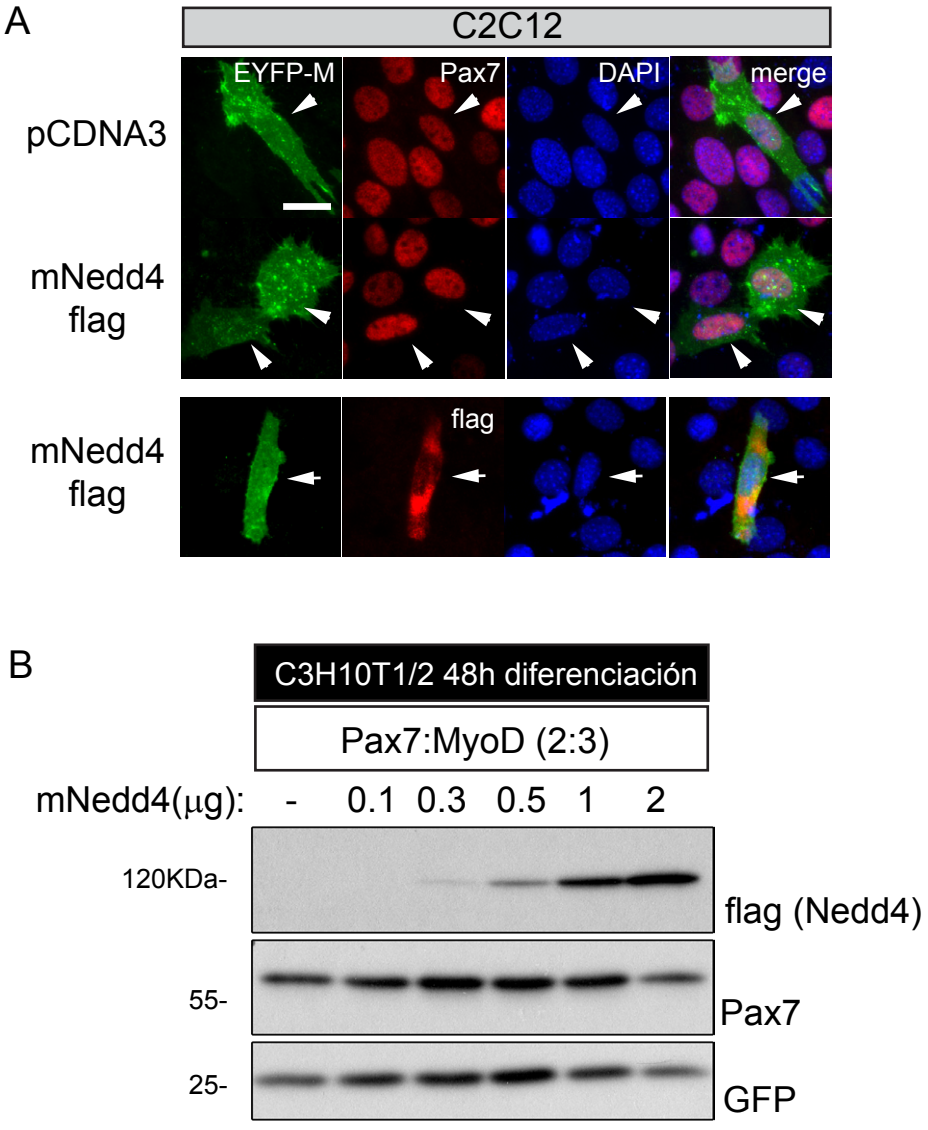
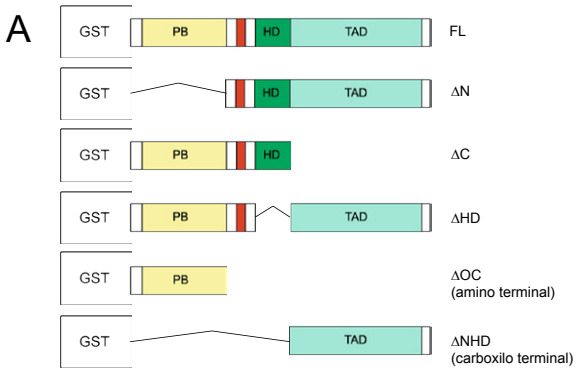
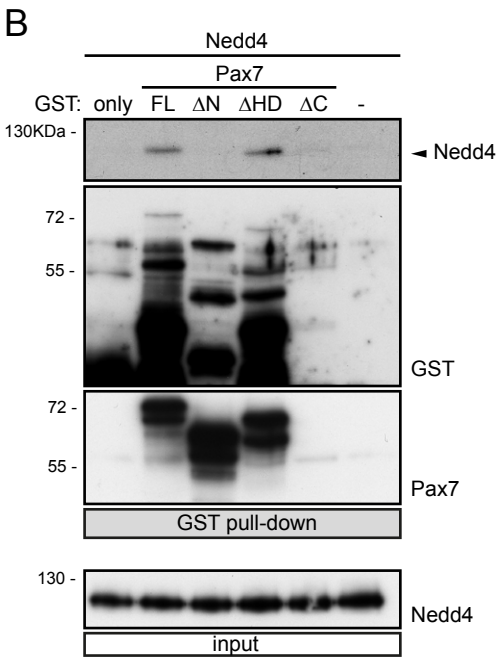


Figura anexa 2





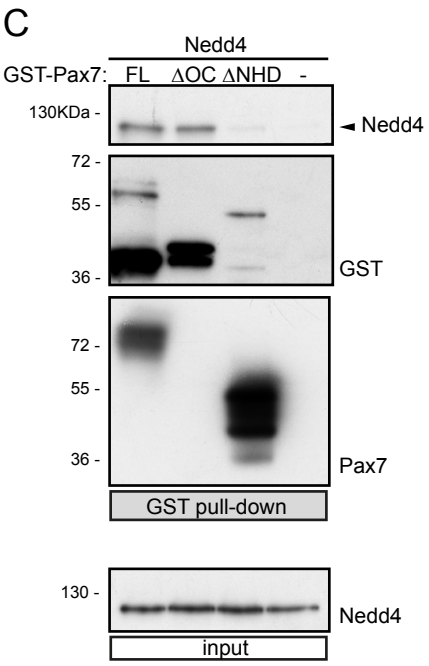


Figura anexa 3